THE TEMPERATURE DEPENDENCE OF CELL CYCLE PARAMETERS AND CHROMOSOMAL DNA REPLICATION IN TISSUE CULTURES OF *Xenopus laevis*

Summary of a thesis submitted for the degree of Doctor of Philosophy, October 1977, by Abdulaziz A. Al-Saleh, Department of Zoology, University of St. Andrews.

Chapter I.

Pulse/chase labelling and DNA fibre autoradiography have been used to study the durations of the stages of the cell cycle, and the manner of chromosomal DNA replication, in *Xenopus* cells in tissue culture at 18°, 23° and 28°C. Cultures were grown in modified Eagle's basal medium, containing salts at concentrations appropriate to Amphibia, plus glutamine and foetal calf serum. The subculturing procedures were carried out every fortnight, with a medium change every week.

For studying the durations of the stages of the cell cycle, cells were labelled with low specific activity tritiated thymidine (^{3}H-TdR) for 30 min or 1 hour, then left to continue growth in non-radioactive medium, and fixed at regular intervals thereafter. Whole-cell autoradiographs, stained in Giemsa, were prepared from these fixations.

For studying DNA replication, tissue cultures were treated with flurodeoxyuridine (FUdR) to arrest cells at the beginning of the S-phase, then labelled with high specific activity ^{3}H-TdR for various times. In the case of pulse/stepdown labelling, the first period of labelling was followed by a further period in the presence of ^{3}H-TdR at one quarter of the original specific activity. DNA fibre autoradiographs were prepared from such labelled tissue cultures.

Chapter II.

An analysis of the durations of the cell cycle stages obtained from pulse/chase labelling experiments gave the following results:- (1) at 18°C G_1 lasts for 31 hrs, S for 29.5 hrs, G_2 for 8.5 hrs, M for 3 hrs and the total generation time is 72 hrs; (2) at 23°C G_1 lasts for 14.3 hrs, S for 15.5 hrs, G_2 for 5.7 hrs, M for 0.5 hrs and the total generation time is 36 hrs, and (3) at 28°C G_1 lasts for 11.3 hrs, S for 13.5 hrs, G_2 for 4.8 hrs, M for 0.4 hrs and the total generation time is 30 hrs.

Pulse labelling followed immediately by fixation, and subsequent Giemsa staining, enables a quick and convenient assessment to be made of the relative durations of the cell cycle stages. In such preparations nuclei in S-phase are labelled, nuclei in G_1 are small and unlabelled and nuclei in G_2 are large and unlabelled.

Chapter III.

Pulse/stepdown labelling shows that DNA replicates bidirectionally in the Xenopus cells. Origin to origin distances (initiation intervals) vary, but the range of and the mean initiation intervals at all three temperatures are much the same. The mean interval between initiation points is of the order of 60 to 66 μm. Staggering of initiations is evident at all three temperatures, and may be disproportionately greater at 28°C than at 23°C and 18°C. Evidence against the existence of replication termini is provided.

Chapter IV.

The rates of progress of DNA replication forks cannot be determined from pulse/stepdown preparations, so these rates had to be estimated from pulse labelled cultures. They are 5.5 μm/hr at 18°C, 10 μm/hr at 23°C and 16 μm/hr at 28°C.

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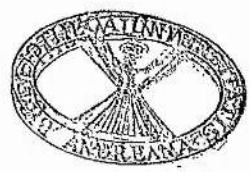

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CHAPTER III.

CHAPTER IV.

CERTIFICATE

I certify that Mr. Abdulaziz A. Al-Saleh has spent 15 terms investigating the cell cycles and chromosomal DNA replication of Xenopus laevis cells in tissue culture at three different temperatures, that he has fulfilled the conditions of Ordinance 16 (St. Andrews), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

H.G. CALLAN M.A. (Oxon)
D.Sc. (Edin.)
F.R.S.

12th October, 1977.

DECLARATION

I declare that this work, which has been carried out on *Xenopus laevis* kidney cells in culture, is my own work and has not been published or previously submitted for any kind of degree.

Abdulaziz A. Al-Saleh.

10.10.1977.

UNIVERSITY CAREER

I entered the University of Riaydh, Saudi Arabia, as an undergraduate in 1969, and three years later was awarded an upper second class honours degree in Zoology and Botany. I worked for the academic year 1972/3 as a demonstrator in the Zoology Department at Riaydh University. I came to the United Kingdom in July, 1973, and spent 3 months studying English before beginning my research in the Zoology Department, University of St. Andrews.

ACKNOWLEDGMENT

I take this opportunity to acknowledge my gratitude and good fortune to have had not only the privilege of study in the United Kingdom, but more especially to have been a student of Professor H.G. Callan, F.R.S. This work has been directed under his supervision, and I wish to thank him for his continuous encouragement, his invaluable and interesting discussion, and his infectious enthusiasm. I am grateful too for his long patience with my English language and writing, and for his guidance in these matters.

My sincere thanks also go to Mrs. L. Lloyd for her continuous help and advice in dealing with tissue culture techniques.

I would like to thank all the staff of the Zoology department for their generous hospitality, and especially to the technical staff for providing equipment and facilities as and when needed. I would also like to thank the secretary of the department, Miss M.M. Moncrieff, for typing my manuscript, and for her help in many other ways.

Finally my thanks go to Riaydh University, Kingdom of Saudi Arabia, for the generous grant made available to me, and to the Saudi Arabian Cultural Bureau in London for their help throughout my stay in the United Kingdom.

GENERAL INTRODUCTION

Unlike cells from animals with constant body temperatures, such as mammals and birds, amphibian cells provide opportunities for the examination of cell cycle durations and features of DNA replication which vary with respect to differing incubation temperatures. Some amphibian cells in culture behave normally over a considerable temperature range, including room temperature (18° - $25^{\circ}C$) and several embryonic and adult amphibian cell lines have been successfully established and maintained *in vitro* (Auclair, 1961; Freed, 1962; Seto, 1964; Wolf & Quimby, 1964; Freed & Rosenfeld, 1965; Balls & Ruben, 1966; Malamud, 1967; Freed *et al.*, 1969; Rafferty, 1969; Freed & Mezger-Freed, 1970, Rudak, 1976). Moreover, several amphibian lines show an unusual ability to maintain a diploid condition, certainly more so than mammalian cell lines (Rafferty, 1969).

The cell line (A-6) derived from the kidney cells of *Xenopus laevis* was primarily chosen for the present study because it is hardy and can be cultured with great ease; it happens to be aneuploid (Rafferty, 1969). Moreover, there exists already some information about its mode of DNA replication at $25^{\circ}C$ (Callan, 1972).

In eukaryotic cells generally DNA replicates during a particular period of interphase (Swift, 1950; Walker & Yates, 1952; Howard & Pelc, 1953; Lajtha *et al.*, 1954). This synthesis period or S-phase is usually preceded by a presynthetic "gap" (G_1) and succeeded by post-synthetic gap (G_2) before the cell goes through its mitotic division (M). The above nomenclature was originally introduced by Howard & Pelc (1953).

The durations of G_1, S, G_2, and M vary during the development of an organism and also vary from one type of cell to another (Defendi & Manson, 1963; Graham, 1966; Graham & Morgan, 1966; Callan, 1973).

Changing the pH or altering the growth medium causes alterations of cell cycle duration mainly by changing the postmitotic period (Sisken & Kinosita, 1961; Sisken & Morasca, 1965; Tobey et al., 1967). The temperature at which cells are grown plays an important part in determining the distributions of the various parts of the cell cycle. An important general point is that all phases of the cycle change, the variation not being restricted to G_1 (Sisken, 1963; Sisken et al., 1965; Rao & Endelberg, 1966; Watanabe & Okada, 1967; Chibon, 1973).

No doubt the S-phases of amphibian cells in culture vary in relation to culture temperature, a matter which I will deal with in this thesis. What is known and remarkable, is that S-phase duration in early newt blastulae (Triturus vulgaris) is of the order 1 to 2 hrs at 18°C, whereas in spermatocytes of the same species and at the same temperature, the S-phase lasts some 200 hrs (Callan & Taylor, 1968; Callan, 1973; 1976). Although the cells of some Amphibia can remain alive over a considerable range of temperature, this does not necessarily mean that they can complete cell cycles throughout the tolerated range; thus Triturus vulgaris larvae can survive at 3°C, but mitoses are arrested at this temperature (Barber & Callan, 1943).

When Watson & Crick (1953) discovered the molecular structure of DNA, they pointed out that DNA replication might be achieved in a semi-conservative manner. The first experimental evidence that DNA replicates semi-conservatively was provided by Taylor et al. (1957), working with root-tips of Vicia faba labelled with tritiated thymidine (^{3}H-TdR). Their autoradiographic findings indicated that cells which had incorporated ^{3}H-TdR during the synthetic period and which were fixed at the immediately following metaphase showed radioactivity in both chromatids of each chromosome. However, cells similarly labelled at one S-phase, but allowed to

pass through mitosis and a second S-phase, this second S-phase being completed in the absence of ^{3}H-TdR, and then fixed at the next metaphase, showed radioactivity in only one chromatid of each chromosome. Their observations not only supported the notion that DNA is replicated semi-conservatively, but also the concept that a chromatid contains only one DNA duplex (i.e. is 'unineme'). Later and similar experimental studies have extended this kind of observation to many other eukaryotic organisms, showing that the two conclusions are generally applicable (Taylor, 1958a; 1958b; 1965; Woods & Shairer, 1959; Filner, 1965; Schwarzacher & Schnedl, 1965; Walen, 1965; Franceschini, 1974). That chromatids of various eukaryotes are unineme in so far as regards their DNA component has also been demonstrated in several other ways (Gall, 1963; Miller, 1965; Kavenoff & Zimm, 1973).

Autoradiographic studies on whole cells have shown that chromosomes do not replicate their DNA sequentially from end to end as a single unit, but instead replicate at many independent regions or sites (Taylor, 1960; Lima-de-Faria, 1961; Painter, 1961; Stubblefield & Muellar, 1962; Moorhead & Denfendi, 1963; Schmid, 1963; German, 1964; Painter, et al., 1966). The most convincing information from whole cells concerning multiple sites of DNA synthesis per chromosome comes from autoradiographic studies of the polytene chromosomes of larval Diptera (Plaut & Nash, 1964; Rudkin, 1972). Both of Plaut (1963) and Mulder, et al., (1968) working with Drosophila, and Pelling (1966) working with Chironomus, have noticed that not all nuclei from salivary glands incubated with ^{3}H-TdR for a brief time are labelled, and of those which incorporated ^{3}H-Tdr, some show whole lengths of the chromosomes uniformly labelled while others show labelling confined to certain regions only. If a chromosome incorporates radioactivity throughout its entire length within as short a period as 10 minutes, this suggests that it must include many sites of

concurrent synthesis, while a chromosome which shows radioactivity in certain regions though not in others again requires that there be at least several sites concurrently active. Mulder et al.'s conclusion was that "many or perhaps all of the replicating units in a salivary gland nucleus start DNA synthesis simultaneously but complete it at different times and data support the hypothesis that the chromomere is a unit of replication or replicon". The first approximation to the number of sites of synthesis in Drosophila salivary gland chromosomes was estimated by Plaut et al. (1966). They incubated salivary glands in tritiated thymidine for 10 to 15 minutes and identified a minimum of 30 sites of synthesis in less than 15% of the length of the X chromosome; this is to say that there are at least some 200 replicating units (replicons) along the four chromosomes of Drosophila if the distribution of replicons in the whole set of chromosomes are arranged uniformly.

Cairns (1962; 1963; 1966) invented an autoradiographic technique which makes it possible to visualize labelled DNA fibres in the light microscope. This technique is based on a gentle dialysis, which aims at obtaining long fibres of DNA from labelled cells. Cairns observations showed that the chromoneme of E. coli consists of one circular double-stranded molecule which replicates from a single "origin", whereas HeLa cell chromosomes replicate from many origins. The same conclusion was reached by Huberman & Riggs (1966) for Chinese hamster cells in culture.

Huberman & Riggs (1968) were the first to show that DNA replication proceeds in opposite directions (bidirectionally) from the origins in hamster cells, and that many origins are arranged in tandem along single DNA fibres. The conclusion that DNA replicates bidirectionally in eukaryotic chromosomes has been confirmed by several investigations

(Amaldi et al., 1972; Callan, 1972; Hand & Tamm, 1972; Weintraub, 1972; Hand & Tamm, 1973; McFarlane & Callan, 1973; Kriegstein & Hogness, 1974; Van't Hof, 1975), and prokaryotic cells are now known to replicate their DNA likewise (Shonös & Inman, 1970; Bourgaux & Bourgaux-Ramoisy, 1971; Jaenich et al., 1971; Masters & Broda, 1971; Bird et al., 1972; Danna & Nathas, 1972; Fareed, et al., 1972; Prescott & Kaimpel, 1972; Cairns, 1973).

Lark, et al. (1971) examined the direction of DNA replication in Chinese hamster chromosomes by using pulse-labelling with low specific activity ^{3}H-TdR followed by another pulse using ^{3}H-TdR of high specific activity and from "streaked" preparations concluded that DNA replication proceeds in one direction, not bidirectionally; this conclusion has been refuted. Huberman & Tsai (1973) re-examined the experiments of Lark et al., by using the same streaking technique (also step-up and step-down labelling) and found that the DNA replication of Chinese hamster chromosomes is certainly bidirectional. They pointed out that unambiguous differences in grain density were obtained only when the differences in specific activity of the isotope at the step were threefold or more. Callan (1973) mentioned that step-up labelling has some technical advantages over step-down labelling for the location of initiation points.

DNA fibre autoradadiography has the merit that it permits one to study not only the arrangement of replication units, but also the rate of progress of replication forks. By this means the rate of fork movement in mammalian cells has been shown to range from 0.2 to 2 μm/min at 37°C (review, Endberg & Huberman, 1975). Callan (1972) working with Xenopus laevis kidney cells, gave an average value for the replication rate as 0.15 μm/min at 25°C.

The aim of the present study has been to compare DNA replication of Xenopus laevis somatic cells in tissue culture at three different incubation temperatures, 18°, 23°, and 28°C. This is to examine how the distribution of initiation points, the rates of replication and cell cycle durations are related to these temperatures.

CHAPTER I.

Materials and Methods

TISSUE CULTURE MATERIALS

Glassware

All bottles, flasks, beakers, measuring cylinders and screw caps used for tissue culture were soaked in a dilute solution of detergent RBS25 (Chemical Concentrates, Limited, London, U.K.) for about one hour, scrubbed well and washed under running water for at least 4 hr. After washing they were rinsed twice with distilled water, twice with triple distilled water, and left to dry in a dust-free place. Bottle necks were wrapped with aluminium foil before they were assembled for sterilization.

Slides were steeped in a cleaning solution (100 gm of potassium dichromate, 100 ml of concentrated sulphuric acid, and water to make up one litre) for 2 hr, washed under running water for several hours, and finally rinsed with distilled water. This method of washing was employed so as to remove contaminants which might otherwise have given background during autoradiography. After washing, slides to be used for DNA fibre preparations were kept in 95% alcohol. Slides for cell cycle analysis were "subbed" with a thin layer of chrome alum/gelatin. These latter were first arranged in a slide holder, and then dipped in subbing solution, then removed and left to dry thoroughly in a dust-free place, subbed slides were used for whole cell autoradiographs when analysing cell cycles, because I found that the emulsion tended to lift from unsubbed slides. The subbing solution consists of an aqueous mixture of 0.1% gelatin and 0.01% chrome alum (chromium potassium sulphate,

$CrK(SO4)_2$, 12 H_2O). The gelatin was first dissolved in boiling water, and the chrome alum was added after the solution had cooled (Gall & Pardue, 1971).

Sterilization

Bottles and Pasteur pipettes were sterilized by dry heat at 160°C for about 4 hr. Screw caps were arranged in a large Petri dish, labelled with autoclaving indicator tape, and autoclaved at 15 lb per square inch for 30 min.

The working place was frequently sterilized by ultraviolet light (Hanovia model 13A), and to prevent contamination the experimental work was carried out under a sterile hood (Bassaire).

All solutions were first prefiltered, then sterilized through large or small Millipore filters of the same pore size, 0.22μ (Millipore S.A. France; SXG 025 0S 0.22). Each filter was examined after use, and if any rupture or a pinhole was noticed, the sterilization was repeated again, using a new filter.

Tissue culture flasks, dishes, pipettes and plastic tubes were obtained as sterile items sealed in plastic bags (Falcon and Becton-Dickinson, U.S.A.).

Media and other solutions

Eagle's basal medium was obtained from Flow Laboratories, Ltd., (U.K.) as sterile solutions of amino-acids (100 x/ml) and vitamins (100 x/ml). Thereafter, both amino-acids and vitamins were divided into 1 ml quantities placed in sterile plastic tubes, and stored at -20°C.

Glutamine (200 mM/ml) was supplied by Flow, Ltd., and divided as required into 1 ml aliquots in sterile plastic tubes, also stored at -20°C.

Penicillin and streptomycin were obtained combined as a sterile powder (DIFCO, U.S.A.). This powder was suspended in 10 ml of sterile triple distilled water, divided into 1 ml quantities in sterile plastic tubes, and refrigerated at -20°C. 1 ml contains 10,000 i.u. of penicillin and 10,000 i.u. of streptomycin.

Foetal calf serum was purchased from Flow, aliquoted into 10 ml quantities in sterile plastic tubes and stored at -20°C.

Amphibian saline with 0.5% lactalbumin hydrolysate was prepared using the ingredients shown in Table 1, dissolved in triple distilled water, sterilized through a Millipore filter (0.22 μm) and placed as 86 ml quantities in 4 oz. sterile bottles. The saline in bottles was kept for a few days at room temperature (18° - 20°C) to check sterility before storing at 4° C.

The growth (or complete) medium was prepared by adding 1 ml each of the amino-acids, vitamins, glutamine, penicillin/streptomycin and 10 ml of foetal calf serum to 86 ml of amphibian saline containing

TABLE 1. Amphibian salts with 0.5% lactalbumin hydrolysate*

Component	gm/litre
NaCl	5.150
KCl	0.075
$MgSO_4$, $7H_2O$	0.204
$Ca(NO_3)_2$ $4H_2O$	0.078
$CaCl_2$	0.045
Na_2HPO_4	0.030
KH_2PO_4	0.037
$NaHCO_3$	0.750
Lactalbumin hydrolysate	5.0
Phenol red (0.5% solution)	2 ml

*Shah. V.C. (1962). modified by the exclusion of yeast extract and the inclusion of phenol red. Phenol red was obtained from Flow as a 0.5% sterile solution.

lactalbumin solution. The complete medium was used fresh and never kept longer than one week.

Trypsin was obtained as a sterile powder (DIFCO) in small vials. The powder in one vial was dissolved in 10 ml of sterile triple distilled water. The resultant enzyme solution has a tryptic activity equivalent to that of a 5% solution of trypsin. This 5% rehydrated enzyme solution was stored in a deep freeze, but for harvesting cells, this solution has to be thawed and then diluted with versene solution to give a 0.025% solution of trypsin.

Versene solution was prepared using the ingredients shown in table 2, dissolved in triple distilled water, sterilized, divided into approximately 100 ml quantities in sterile bottles, and stored at room temperature. Cells which are to be used for subculture have to be rinsed once or twice with a balanced salt solution containing versene so that they will detach from the surface of the tissue culture flask when trypsinized. Culture medium contains serum, which inactivates trypsin (Priest, 1969); the versene is used to remove serum in the culture medium and the divalent cations calcium and magnesium, which are involved in cell adhesion.

Thymidine (TdR 500 µg/ml), 5-fluorodeoxyuridine (FUdR, 100 µg/ml) and uridine (UR 50 µg/ml) were separately dissolved in triple distilled water, sterilized through Millipore filters (0.22 µm) 9nto sterile plastic tubes, and stored at -20°C until required.

TABLE 2. Versene solution

Component	gm/litre
Na_2-EDTA	0.200
NaCl	6.0
KCl	0.2
Na_2HPO_4	1.065
Phenol red (0.5% solution)	1.0 ml

SUBCULTURE PROCEDURES

I have worked with an established cell-line (A-6) of Xenopus laevis originally set up by Dr. K.A. Rafferty. A subculture came from Glasgow to the Zoology Department, St. Andrews University, in October 1969, and has been maintained there since then. The subculturing technique described below was used routinely once a fortnight, with a medium change once per week.

The flask containing confluent cells to be subcultured was selected, and the old growth medium discarded. The cell monolayer was rinsed once with about 5 ml of versene solution, and then the versene rinse was poured off. About 2.5 ml of 0.025% trypsin solution was added to the flask and the flask agitated manually for approximately 5 minutes until all the cells had detached from the surface of the tissue culture flask. Thereafter, 5 ml of fresh medium was added to the flask in order to suppress the action of the trypsin, and the cell suspension transferred to a sterile plastic tube. The cells were centrifuged at 600-800 r.p.m. for about 5 min. using an MSE Minor centrifuge. The supernatant was pipetted off using a sterile Pasteur pipette, and the pellet of cells resuspended in 5 ml of fresh medium. 1 ml of the cell suspension was now added to a new 75 cm^2 plastic tissue culture flask containing 15 ml of fresh medium, and the flask incubated at the required temperature (18°, 23°, or $28^{\circ}C$), in a water jacketed incubator. The pH was adjusted, if necessary, with 5% CO_2 and 95% air to give the appropriate colour (pink-orange) at the proper pH of 7.4.

METHODS USED FOR CELL CYCLE ANALYSIS

Following the frequency of labelled mitoses with time, after pulse-labelling and a cold chase, is one of the most widely used methods for studying cell cycle duration. This technique was first used by Howard & Pelc (1953), and further developed by Quastler & Sherman (1959).

Setting up the subcultures

A flask containing near confluent cells was selected, and trypsinized according to the subculture procedures outlined on page (10). The cell suspension was centrifuged, and the pellet of cells gently resuspended in 5 ml of fresh medium. The cell concentration was estimated by a haemocytometer count, and about 5×10^5 cells/ml of cell suspension added to 5 ml of fresh medium in each of seventeen 25 cm^2 plastic tissue culture flasks. The replicate subcultures were left to enter the logarithmic growth phase, for approximately 96 hrs at 18°C, and for 48 hrs at 23°C and 28°C. When cells had reached the log phase, 10 µC of tritiated thymidine (^{3}H-TdR, 5 Ci/mM; Radiochemical Centre, Amersham) was added to each flask, left for 30 min at 23°, and 28°C, and for 1 hr at 18°C, using the appropriate safety measures. After labelling, the radioactive medium was poured off and the cells washed twice with non-radioactive medium. 5 ml of a new medium, containing non-radioactive thymidine (TdR) at 100 times the molarity present in the previous radioactive medium, was added to each flask and incubated at the required temperature. The aim of adding TdR to the medium was quickly to dilute the pool of labelled molecules and hence stop further incorporation of label beyond the intended labelling period (Cleaver, 1967). At three-hour intervals in the case of the 23°C and 28°C cultures, and at six-hour

intervals for the 18°C cultures, one flask was removed, trypsinized, the contents removed and the pellet of cells resuspended in 0.5 ml of 0.075 M potassium chloride for hypotonic treatment. The cells were fixed directly on subbed slides with a 3:1 mixture of absolute alcohol and glacial acetic acid. A small drop of the cell suspension was dropped on to a subbed slide wetted with the fixative; after a few seconds more fixative was added at one end of the slide, which had the effect of driving away the drop of hypotonic solution, leaving a circle of cells attached to the middle of the slide. The free fluid was taken up with a Kleenex tissue, and the slide left to dry for one day or more.

Whole cell autoradiography

The dry preparations were coated with Kodak NTB2 emulsion (Kodak, Ltd., U.K.) diluted to one half of its original strength with distilled water. Under a safelight (Wratten, series 1 and 15 watt bulb), the emulsion vial was first warmed at 45°C in a temperature controlled water bath for about 5 minutes, and a suitable amount of emulsion transferred to a small flattened "dipping" vial which was mounted in the water bath. The slides were then dipped into the emulsion each for about 5 seconds, then withdrawn slowly and steadily in order to produce a thin uniform layer of the emulsion on the slide. The filmed slides were stood upright in racks in front of a fan powered by a bnushless motor, and left to dry thoroughly. Thereafter they were arranged in light-tight boxes, sealed with self-adhesive strips, packed in a large cardboard box, and left to expose fof 5 to 6 days in a refrigerator at 4°C. Exposure times of 5 to 6 days were found to be the optimum for clearly distinguishing labelled cells from unlabelled cells. Exposure times of less than 5 days

gave inadequate silver grain densities over labelled cells, while exposures of much more than 6 days often gave cell nuclei entirely concealed by silver grains. The exposed slides were developed for $2\frac{1}{2}$ minutes in total darkness at 20°C with Kodak D19 (Kodak, Ltd., U.K.), rinsed in distilled water, and fixed for about 3 minutes with Kodak Unifix fixer. The preparations were now washed for about 5 minutes under slow running filtered tap water and finally rinsed in distilled water. Some preparations were stained immediately, others air-dried until the time of staining.

Staining

The preparations were stained for 1 hr by putting three slides in a Coplin jar containing 50 ml of 0.01 M phosphate buffer (pH 6.9), then adding 2 ml of Giemsa staining solution (BDH Chemical, Ltd., Pool, England) and mixing the fluids by pipette or gentle agitation in the Coplin jar. This was done to avoid the scum problem inherent in all Giemsa staining techniques. After 1 hr the staining solution was washed away under a gentle stream of distilled water, and the slides left to dry ready for microscopic examination. These slides were not covered with coverslips.

Recording the results

Labelled and unlabelled metaphases were counted, and the percentages of labelled metaphases were plotted on a graph against time. The cells were sampled at random by scanning entire lengths of a slide an appropriate number of times, and no less than 200 metaphases were examined for the determination of each point on the graph.

Stained whole cell autoradiographs were photographed using Ilford Pan F film. The microscope was arranged with 40x oil immersion objective, 1.6x optovar, and 5x eye piece. A Zeiss broad band interference filter was used, and a "spreading lens" inserted below the condenser to give full illumination of the field being photographed. Exposure times were 2 to 3 seconds. Examples of metaphase chromosomes were photographed using a 100x oil immersion objective.

METHOD USED FOR DNA FIBRE AUTORADIOGRAPHS

The technique originally devised by Cairns (1962; 1963; 1966), with more detailed instructions by Huberman & Riggs (1966; 1968), and with a few modifications introduced by Callan (1972), was used in the present study.

Subculturing and labelling procedures

Xenopus laevis cells were subcultured as usual (page 10) and incubated at the required temperatures (18°, 23°, and 28°C). A week later the medium was replaced by fresh medium and the cells left to grow for another week. A flask containing near confluent cells was selected, the monolayer of cells was trypsinized, harvested, and centrifuged, and the pellet of cells suspended in 5 ml of fresh medium. Thereafter, Petri dishes (60 mm in diameter) were set up with about 2×10^5 cells/dish. Each dish contained 4 ml of fresh medium. The dishes were then arranged inside a desiccator, with a small quantity of distilled water in the bottom to maintain humidity. The pH was adjusted where necessary with 5% CO_2 and 95% air. The desiccator was sealed and incubated for 48 hours at 28°, 23°C and twice as long at 18°C. When cells appeared to be

near confluence, 5-fluorodeoxyuridine (FUdR) and uridine (UR) were added to each dish to give a final concentration of 1 µg/ml of FUdR and 0.5 µg/ml of UR, and the dishes were incubated for a further 20 hr at 28° or 23°C and for a further 40 hr at 18°C. This step was applied to arrest most of the cells at the beginning of S-phase (Callan, 1972). After the treatment with FUdR/UR, the medium was poured off from all dishes except two, which were kept as controls for cell counts (one had been treated with FUdR/UR and the other not). Thereafter 2 ml of fresh medium containing 50 µC/ml of ^{3}H-TdR (26 Ci/mM) were added to each dish and the cells were labelled as follows:

1. Subcultures grown at 28°C:-

The cells were labelled for 1 hr, 2 hrs, 4 hrs, and for 1 hr followed by a 1 hr stepdown. The stepdown was performed by adding sufficient non-radioactive thymidine (TdR) to the original medium so as to reduce the specific activity of the radioactive thymidine to one quarter of its original level.

2. Subcultures grown at 23°C:-

The cells were labelled for 2 hrs, 4 hrs and for 2 hrs followed by a 2 hrs stepdown. The stepdown was carried out by reducing the specific activity of ^{3}H-TdR just as described above.

3. Subcultures grown at 18°C:-

The cells were labelled for 2 hrs, 4 hrs, 8 hrs and for 4 hrs followed by a 4 hrs stepdown.

After labelling, the radioactive medium was pipetted off by Pasteur pipette, the cells were rinsed once with 2 ml of versene solution containing

1 µg/ml FUdR (to suppress further DNA synthesis) and then the versene solution removed. The cells were harvested with 1 ml of 0.25% of trypsin in versene solution also containing 1 µg/ml FUdR, the dishes being incubated until all the cells had detached from the surface (usually the dish has to be agitated manually but gently in order that the cells detach more quickly). The cell suspension was collected in a plastic tube, the tube dipped into crushed ice, and according to the cell number present in the control dish which had been treated with FUdR/UR, the cells were diluted to the appropriate range of concentrations from 1×10^5 to 0.6×10^4 cells/ml with sucrose medium (1M sucrose, 0.05 M NaCl, 0.01 M EDTA, pH adjusted to 8 with NaOH).

Dialysis chambers

The dialysis chambers were constructed from Pyrex glass rod of 4 mm thickness, and Pyrex glass tubing of 4 mm O.D. and 2 mm I.D. The glass rod is heated, fused to one end of a 12 cm long piece of tubing, bent to form a ring of about 25 mm O.D. and 17 mm I.D., and the other end of the rod likewise fused back to the piece of tubing. When cool, flat surfaces are ground on both sides of the ring (text-fig. 1, I & II). To each side of the ring 25 mm diameter Millipore filters (VM 50 mµ) are attached with Durofix adhesive (Rawlplug Co. Ltd., U.K.), the glossy surface of the filter placed to the inside of the chamber (text-fig. 1, III). The chambers are left to dry out (but not baked) for several days prior to use.

Dialysis procedure

The serial dilutions (1×10^5; 5×10^4; 2.5×10^4; 1.25×10^4; and two lots of 6×10^3) of the cells in sucrose medium were separately

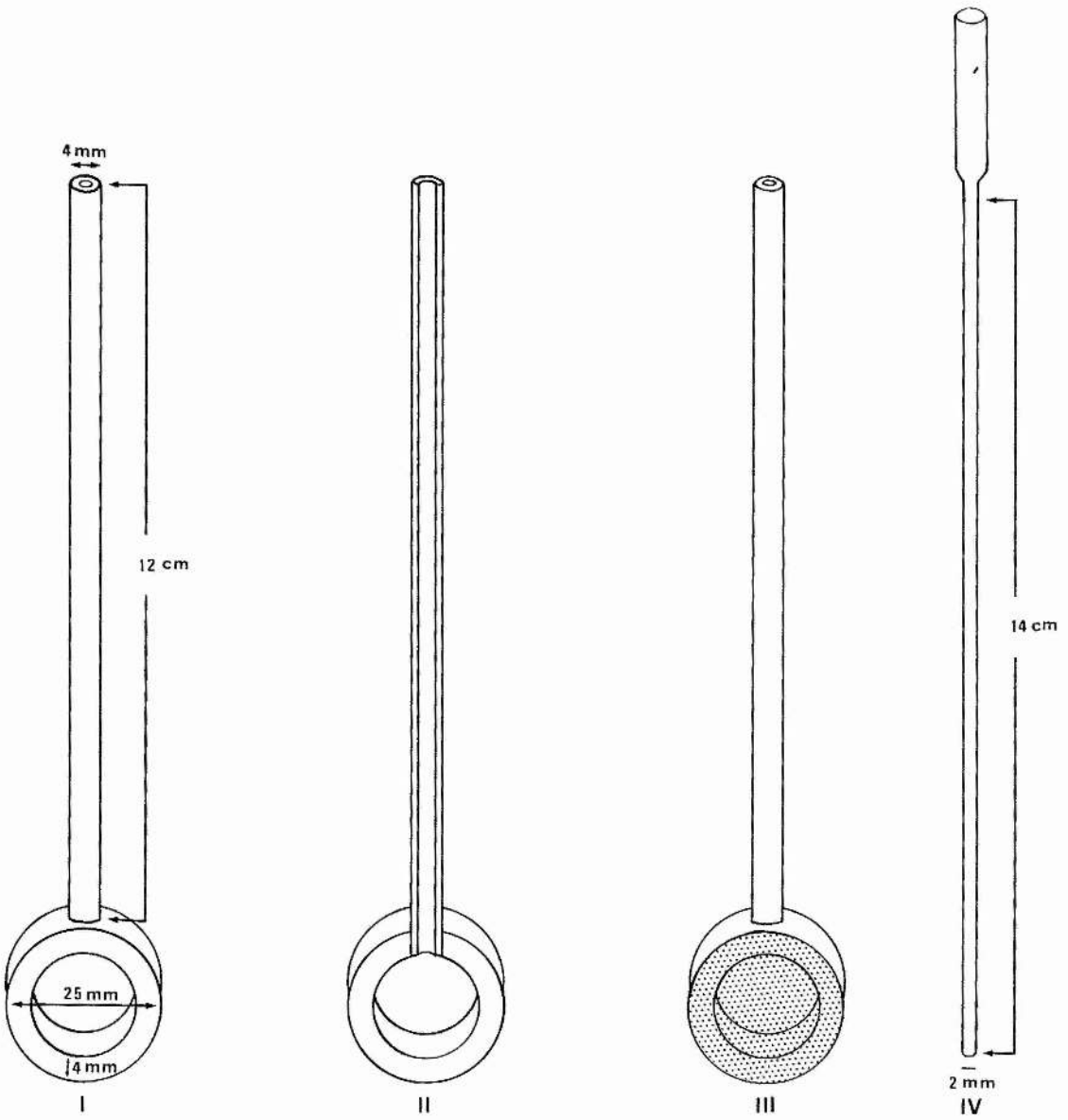
Text-fig. 1 is an illustrative drawing of the dialysis chamber chamber according to Callan's description (1972). The chamber is constructed from a ring of Pyrex glass rod fused to a stem of Pyrex glass tube, with flat surfaces ground on the ring.

I. - Three dimensional view of the chamber.

II. - Longitudinal section of the chamber.

III. - The chamber with the Millipore filter.

IV. - The fine-drawn pipette used for filling dialysis chambers.


4 mm
12 cm
25 mm
4 mm
I
II
III
14 cm
2 mm
IV

transferred, 1 ml at a time, using thin long siliconized pipettes, to the dialysis chambers; each chamber was filled gently from the bottom upwards in order to avoid air bubbles forming inside the chamber. The chambers containing cell suspensions were labelled, their stems inserted in holes in a flat sheet of cork, and the cork sheet placed above a glass vessel (text-fig. 2) containing the dialysis solution, with the chambers dipping into this solution. The glass vessels containing dialysis medium were arranged inside a constant temperature bath adjusted to 25°C.

The cells were first dialysed against 1% SDS (sodium dodecyl sulphate) in sucrose medium for about 4 hrs. SDS is a detergent which destroys biological membranes, allowing the nuclear contents to come free. The viscous sucrose solution both inside and outside the chambers acts to prevent violent fluid movements and hence minimizes shear breakage of the chromatin fibres as these become released from cell nuclei (Huberman & Riggs, 1966; Callan, 1972; 1973). If any air bubble was noticed in the stem of a dialysis chamber, the fluid above the bubble was removed by a fine pipette in case it contained cells which has not been subjected to detergent action. After dialysis against SDS/sucrose for 4 hrs the assembly of chambers on a cork sheet was lifted and placed over another vessel containing saline-versene (0.05 M NaCl, 0.01 M EDTA, pH brought to 8 with 1 N NaOH). Dialysis against saline-versene, the purpose of which is to remove SDS, was continued for one hour.

The cells were then dialysed against 1 mg/ml of "matured" pronase (Sigma-protease) in SSC-tris (0.15 M NaCl, 0.015 M sodium citrate, 0.01 M tris, pH adjusted to 8 with 1N NCl) overnight. The matured pronase (the solution was made up and left at 37°C for 2 hrs so that any possible DNase contaminant was digested) hydrolyses proteins, and in this manner

Text-fig. 2 is an illustrative drawing of the arrangement of dialysis chambers inside a dialysing vessel.

C.L., a cork lid.

D.C., a dialysis container.

C.CH., a dialysis chamber.

M.F., a Millipore filter.

M.F.
D.CH.
D.C.
C.L.

DNA fibres in the dialysis chambers become freely extended. Following pronase digestion, the chambers were dialysed against saline-versene medium, 4 changes, each of 2 hrs, in order to remove the pronase and the products of its digestion from the chambers.

The assembly of dialysis chambers was now removed from the final dialysis solution, held upright, and again if any solution happened to be trapped above an air bubble in the stem of a chamber it was removed with a fine pipette. Each individual chamber was removed from the cork support, and one of its Millipore filters was punctured at the bottom with a fine needle. The contents were allowed to drain out slowly and steadily on to a filter paper. The chambers were now left to dry at room temperature overnight. The Millipore filters were cut away or prised from the chambers and trimmed with a fine pair of scissors, cutting away that portion which had originally been attached to the ground glass surround. The trimmed filters were placed, glossy side down, on a filter paper, their sources of origin recorded, and they were now exposed to concentrated neutral formalin vapour for 2 hrs. The formalin chamber consisted of a bowl with a small pad of cotton wool attached to its bottom, the pad being liberally wetted with formalin. The reason for exposing the filters to formalin is to denature any residual pronase which might otherwise digest the AR10 film later to be applied (Huberman & Riggs, 1968). Finally each filter was stuck glossy side up on to a clean labelled slide at 4 places with minute drops of Durofix adhesive, and left in an open box overnight.

Autoradiographic procedure

Slides bearing Millipore filters were now filmed with Kodak AR10 stripping film. The filming was carried out in a dark room using a red safelight (Wratten series 1) with a 15w bulb. Kodak AR10 stripping film (Kodak, Ltd., U.K.) was cut into rectangular pieces approximately 3" x 1", large enough to cover the Millipore filter and to overlap at the back of the slide, then peeled off gently with a pair of forceps and dropped, sensitive side down, on to a glass dish containing distilled water at 20°C. After allowing each piece of film to absorb water and expand for 1 or 2 min, the slide was pushed slantwise under the film, then lifted out with film draped over, and the slide twisted to left and right so as to overlap the ends of the film on one another at its back (see Rogers, 1967). The covered slides were left to dry in front of a fan powered by a brushless motor for half an hour, then packed in light-tight boxes, and the boxes sealed with self-adhesive tape to prevent air and light leakage. The boxes were arranged in a large cardboard box, labelled and dated, and the slides left to expose for 8 to 11 months in a refrigerator at 4°C.

Exposed slides were developed in total darkness for 20 min with Kodak D19 at 20°C, only absolutely fresh developer being used. After development the slides were rinsed twice in distilled water and fixed in Kodak Metafix for about 5 min. The slides were now arranged in individual Petri dishes and washed under gently running filtered tap water for 10 min before being rinsed in distilled water. Each of the slides, one by one, was transferred to a large dish containing distilled water, and under a binocular dissecting microscope the back of the film was cut with fine scissors. The slide was now reversed and gently

joggled until the film, together with its Millipore filter, separated from the slide. By grasping the Millipore filter with one pair of forceps and the film with another the film was gently peeled off, and left submerged with its sensitive side down. A clean labelled slide was now passed below the film and lifted out of the dish, carrying the film, the aim being to trap the film so that the region previously in contact with the Millipore filter came to lie in the centre of the slide. This operation has to be done reasonably rapidly in order to prevent undue stretching of the film. The edges of the film were overlapped at the back of the slide, and the slide left to dry completely in a rack over a warm plate. Dry slides were now immersed in 2% formalin for 10 min to harden the film, then washed in gently running filtered tap water for at least 10 min, rinsed in distilled water, and the film at the back of the slide carefully scraped off. Finally the slides were left to dry thoroughly so as to be ready for microscopic examination. This elaborate washing procedure was introduced so as to prevent excessive stretching of AR10 film during processing, some batches of the film being particularly prone to stretching. The overlapped film at the back of the slide traps both developer and fixative, and both must be washed out. After the first, inadequate, wash the film is dried in position on the slide, and the formalin treatment rigidifies it in this state. Thereafter the washing can be prolonged without danger of further film stretching.

Microscopy and measurement

DNA fibre autoradiographs were examined with a Zeiss microscope by bright-field illumination. Using a 40x planapochromat oil immersion objective and 12.5x eye pieces, the autoradiographs were identified as tandemly arranged silver grain tracks. The lengths of labelled tracks

and the lengths of initiation intervals were measured in eye piece units with a focussing 12.5x eye piece. The microscope was set with its optovar at 1.25, and so arranged gave a magnification where 50 divisions of the eye-piece scale were equal to 100 μm. After examination, immersion oil was removed from a slide by washing it in two changes of petroleum ether followed by a rinse in acetone. Xylene must not be used to remove immersion oil, because it causes the very large silver grains produced by long development time to turn yellow and fade.

Photographs of DNA fibre autoradiographs were taken under bright-field illumination (40x oil immersion objective, 5x eye piece) using 35 mm Pan F film (Ilford Ltd., U.K.). With the light appropriately adjusted, exposure time was between 3 and 4 seconds. The negatives were developed with ID2 for 6 min at 20°C, washed twice with filtered tap water, fixed with Amfix for 10 min, washed under running water for half an hour or more, rinsed with distilled water and suspended until dry. Photographic prints were made using Ilford hard paper grade no. 3 at enlargement giving a final magnification of 500x (50 mm = 100 μm).

CHAPTER II.

Temperature and the Xenopus cell cycle

INTRODUCTION

Variation in the temperature to which mammalian cells are exposed has an effect on all parts of the cell cycle (Sisken et al., 1965; Rao & Engelberg, 1966; Watanabe & Okada, 1967). There is not much information in the literature concerning the relationship between cell cycle duration and temperature in Amphibia. However in the case of the stage 34 embryo of Pleurodeles waltlii it has been shown, for example, that in fore-limb mesenchyme cells the S-phaseslast for 79 hrs at 12°C, and for 14 hrs at 26°C (Chibon, 1973).

The aim of the present study has been to determine the durations of all parts of the Xenopus somatic cell cycle in tissue cultured cells at 3 different temperatures by means of the labelled mitoses method.

METHODS AND RESULTS

The experiments were carried out on an established cell line (A-6) of Xenopus laevis originally produced by Dr. K.A. Rafferty (1969). The procedures used for culturing cells and determining the durations of the parts of the cell cycle have already been described in chapter I, Materials and Methods (p. 11). In outline, the cells were grown at 18°C, 23°C, and 28°C in a complete amphibian medium. Subcultures were started with 5×10^5 cells/5 ml of medium in 25 cm^2 plastic tissue culture flasks. Replicate subcultures at 23°C and 28°C were left for 48 hrs to reach logarithmic growth, while subcultures grown at 18°C were left for 4 days. Cells in the log phase at 23°C and 28°C were labelled with 2 µC/ml of

^{3}H-TdR (5 Ci/mM) for 30 min, while those at 18°C were labelled for 1 hr. After labelling, the radioactive medium was poured off, cells washed twice with non-radioactive medium, and then 5 ml of fresh medium containing TdR at 100 times the molarity of the previous ^{3}H-TdR medium was added to each flask and incubation continued.

At 3-hour intervals (6 hours in the case of cultures at 18°C) one flask was removed, the cells were trypsinized, centrifuged, and the pellet of cells resuspended in 0.076 M KCl. The cells were fixed directly on slides with 3 parts absolute alcohol to 1 part glacial acetic acid, and air dried. The slides were filmed with NTB2, exposed for 5 to 6 days, developed, and stained for one hour with Giemsa. Using these preparations, labelled and unlabelled metaphases were scored and the percentage of labelled metaphases plotted against time.

Following the frequency of labelled mitoses after a pulse-chase labelling procedure is one of the most satisfactory methods for determining not only the duration of the generation time, but also the durations of G_1, S and G_2. The method is based on labelling cells with tritiated thymidine, and then chasing with a medium containing non-radioactive thymidine in order to restrict labelling to a brief interval only (Cleaver, 1967). Cells which are in the S-phase during the pulse will be labelled, and if these cells are allowed to continue growth, the labelled cells will pass through mitosis. By sampling cells at intervals and estimating the appearance and disappearance of labelled mitoses in autoradiographs (see figs. 1 to 5) a succession of waves of labelled mitoses can be followed.

Tables 3, 4 and 5 show the data which I obtained from pulse-chased Xenopus cultures, and this information is expressed graphically in text-figure 3.

TABLE 3. Percentage of labelled metaphases derived from Xenopus laevis cells in subcultures at 18°C labelled with ^{3}H-TdR for 1 hr and fixed at 6 hour intervals.

Time after addition of label (hr)	Labelled metaphases / Total metaphases	% of labelled metaphases	Time after addition of label (hr)	Labelled metaphases / Total metaphases	% of labelled metaphases
6	065/389	17	54	042/244	17
12	230/410	56	60	042/254	16
18	190/260	73	66	066/378	17
24	312/378	82	72	075/330	23
30	167/228	73	78	118/366	32
36	200/358	56	84	154/388	40
42	078/250	31	90	206/402	51
48	072/334	21	96	210/378	55

TABLE 4. Percentage of labelled metaphases derived from Xenopus laevis cells in subcultures at 23°C labelled with ^{3}H-TdR for 30 minutes and fixed at 3 hour intervals.

Time after addition of label (hr)	Labelled metaphases / Total metaphases	% of labelled metaphases	Time after addition of label (hr)	Labelled metaphases / Total metaphases	% of labelled metaphases
3	015/370	04	27	060/412	15
6	174/357	49	30	037/310	12
9	282/318	89	33	051/279	18
12	335/365	92	36	058/264	22
15	311/355	88	39	104/336	31
18	230/310	74	42	144/320	45
21	187/375	50	45	125/241	52
24	111/393	28	48	122/262	47

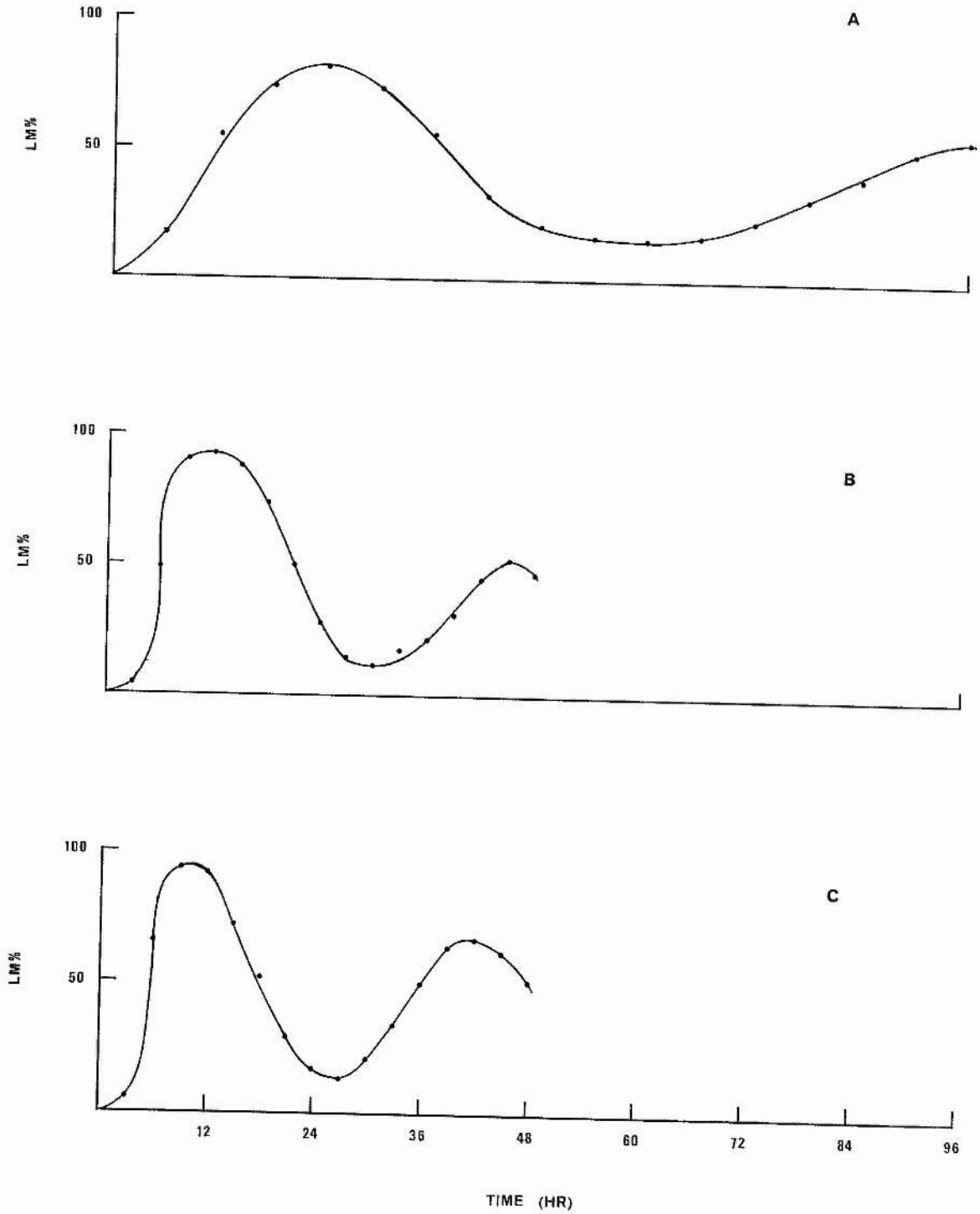
TABLE 5. Percentage of labelled metaphases derived from Xenopus laevis cells in subcultures at 28°C labelled with ^{3}H-TdR for 30 minutes and fixed at 3 hour intervals

Time after addition of label (hr)	Labelled metaphases / Total metaphases	% of labelled metaphases	Time after addition of label (hr)	Labelled metaphases / Total metaphases	% of labelled metaphases
3	016/266	06	27	030/230	13
6	234/348	67	30	068/320	21
9	308/328	94	33	114/336	34
12	296/326	91	36	195/384	50
15	294/408	72	39	268/420	64
18	114/226	50	42	256/372	69
21	141/483	29	45	186/304	61
24	045/266	17	48	183/357	51

Text-fig 3 plots the percentages of labelled metaphases in Xenopus kidney cells in culture at the three different culture temperatures, 18°C (3A), 23°C (3B) and 28°C (3C) as a function of time. These curves show that the proportions of labelled metaphases rise to a peak, then descend through a trough, and rise to a peak again. The first ascending portion of the curve is a consequence of cells which were labelled at progressively earlier stages of the S-phase passing through G_2 and reaching mitosis. Theoretically the labelled frequency should reach 100%, but in practice this is not observed because of some cell to cell variability in cycle durations. The descending portion of the curve is a consequence of cells which were in progressively earlier stages of G_1 at the time of labelling passing through the S and G_2 phases and reaching mitosis.

The time interval between the ordinate and mid-point of the ascending curve represents the duration of $G_2 + \frac{1}{2}$ M (where M = time spent in mitosis). Half the mitotic time is added to G_2 because the mitoses are scored only in metaphase, which means that cells have to be through not only G_2 but also prophase. The width of the first wave from the mid-point of the ascending curve to the mid-point of the descending curve measures the duration of the S-phase. The duration of the cell cycle, or generation time (Tg) is measured by the time interval between the first and second ascending curves, both taken at their mid-points. The duration of G_1 and the other half of the mitotic time may be determined by subtracting the total duration of $G_2 + \frac{1}{2}$ M plus S from the generation time (Mitchison, 1971).

Text-fig. 3 shows the percentages of labelled metaphases as a function of time, obtained from whole-cell autoradiographic preparations of Xenopus cells cultures at 18° (A), 23° (B) and 28°C (C). See text for further details.


100
50
LM%
A
100
50
LM%
B
100
50
LM%
C
12
24
36
48
60
72
84
96
TIME (HR)

Temperature and generation time

The data relating generation time to culture temperature is summarized in Table 6. This table has been constructed from the curves shown in Text-fig. 3, the curves themselves having been drawn from the raw data presented in tables 3, 4 and 5. As will be seen in table 6, generation times at 18°, 23°C and 28°C are 72, 36, and 30 hrs respectively.

There is no firm information as to whether amphibian cells in culture have an optimum culture temperature; Freed & Mezger-Freed have mentioned in their review (1970) that amphibian cells (Rana pipiens) in culture grow rapidly at 25°C but that temperatures above 28°C are injurious, although this effect may only be manifest after a delay. Seto & Rounds (1969) point out that cultures of frog (Rana nigromaculata) kidney cell strain and newt (Taricha torosa) lung cells grow best at 26°C, are retarded below 22°C, and inhibited at 37°C.

Rafferty (1969). who originally established the Xenopus A-6 kidney cell line, cultured his cells at 25°C and found them to have a generation time of about 22 hrs.

I do not know whether my Xenopus A-6 cells still have a generation time of 22 hrs at 25°C; they may have altered in the course of culture. I therefore cannot state with assurance that the optimum temperature for the culture of the Xenopus A-6 cell line is 25°C, nor whether the relatively trivial difference in generation times at 23°C and 28°C is a consequence of 28°C being above the temperature optimum for these cells.

TABLE 6. The durations of cell cycles and their component phases determined from pulse-labelled preparations obtained from Xenopus cells in culture at 18°, 23° and 28°C.

Temperature °C	$G_1 + \frac{1}{2}$ M hr	S hr	$G_2 + \frac{1}{2}$ M hr	Tg hr
18	32.5	29.5	10	72
23	14.5	15.5	6	36
28	11.5	13.5	5	30

G_1 = pre-synthetic period.

S = synthetic period.

G_2 = post-synthetic period.

M = mitosis

Tg = generation time.

Temperature and S-phase duration

The data relating S-phase duration to temperature is summarized in Table 6. At 18°C the S-phase lasts for 29.5 hrs, at 23°C for 25.5 hrs and at 28°C for 13.5 hrs.

J.H. Priest and J.A.M. Cooper (unpublished; quoted in Callan, 1972) give an S-phase duration of the order 13 hrs at 25°C. My own data coupled with that of Priest and Cooper would suggest that the optimum temperature for the Xenopus kidney cell line must be in the neighbourhood of 25°C.

Temperature and the duration of G_1, G_2 and M.

Table 6 shows the durations of G_1 plus one half M to be 32.5 hrs at 18°C, 14.5 hrs at 23°C and 11.5 hrs at 28°C. Likewise, the durations of G_2 plus one half M are 10 hrs at 18°C, 6 hrs at 23°C and 5 hrs at 28°C.

Mitotic indices were determined for the cells growing at all three temperatures, and these are shown in Table 7. One can calculate the approximate duration of mitosis from the formula:- Time in mitosis = 1.44 x mitotic index x generation time, provided mitotic time is only a small fraction of generation time (Hughes, 1952).

It will be seen from Table 7 that mitosis takes roughly 3 hrs at 18°C, but only half an hour at 23° and 28°C. With this information one can assess the durations of G_1 and G_2 more precisely, and these are given in Table 8. At 18°C G_1 lasts 31 hrs, at 23°C it lasts 14.3 hrs and at 28°C 11.3 hrs. At 18°C G_2 lasts 8.5 hrs, at 23°C it lasts 5.7 hrs and at 28°C 4.8 hrs.

In principle it ought to be possible to determine the relative durations of G_1, S, G_2, and M directly from autoradiographs of cells in tissue culture

TABLE 7. The durations of mitosis in Xenopus cells cultured at 18°, 23° and 28°C.

Temperature °C	No. of mitoses / Total no. of cells	Mitotic index MI	Generation time Tg (hr)	Mitotic time M = Tg x MI x 1.44 (hr)
18	29/1000	0.029	72	3.00
23	26/2667	0.010	36	0.50
28	13/1264	0.010	30	0.40

TABLE 8. The absolute and relative durations of the component phases of Xenopus tissue culture cell cycles at 18°C, 23°C and 28°C after adjustment of the G_1 and G_2 phases for the time spent in mitosis.

Cell cycle parts	18°C	23°C	28°C
$G_1 + \frac{1}{2}$ M	32.5 hr	14.5 hr	11.5 hr
G_1	31.0	14.3	11.3
G_1 as a function of $T^a g$	0.43	0.40	0.38
G_1 as a function of $T^b g$	0.34	0.34	0.32
S	29.5	15.5	13.5
S as a function of $T^a g$	0.41	0.43	0.45
S as a function of $T^b g$	0.43	0.48	0.49
$G_2 + \frac{1}{2}$ M	10.0	6.0	5.0
G_2	8.5	5.7	4.8
G_2 as a function of $T^a g$	0.12	0.16	0.16
G_2 as a function of $T^b g$	0.18	0.15	0.17
M	3.0	0.5	0.40
M as a function of $T^a g$	0.04	0.01	0.01
M as a function of $T^b g$	0.04	0.01	0.01
Tg	72	36	30

Tg = generation time

a = computed from the cell cycle analysis

b = computed from adjusted cell counts in autoradiographs.

which have been pulse-labelled with ^{3}H-TdR. Those cells which are labelled represent cells in S, and of the unlabelled cells there should be two classes distinguishable by their DNA content, G_1 cells with 2C and G_2 cells with 4 C. Again, in general terms, G_1 nuclei should be distinctly smaller and separable from G_2 nuclei. In practice there are problems. Variable degrees of flatness of cell nuclei in autoradiographs, and cells of ploidy higher than diploid, complicate the picture.

In spite of these difficulties I have attempted to analyse pulse-labelled autoradiographs along these lines. A series of slides were prepared and stained by the Feulgen technique; these proved to be valueless for lack of sufficient stain in the nuclei. In order to intensify the staining, a modified Feulgen technique devised by Dr. H. Swift (personal communication with Prof. H.G. Callan), where Azure A is employed instead of Schiff's reagent, following the standard hydrolysis in normal HCl, was tried out. This was abandoned because of the high film background grain counts generated by Azure A staining. In the end I decided to work with slides stained with Giemsa, attempting to discriminate between G_1 and G_2 cells on the basis of nuclear size rather than DNA content. This proved to be practicable, at least to a first approximation, by confining attention to small clusters of cells within which the degree of flattening of the cells appeared to be uniform (see figs. 1 to 5).

The raw data obtained in this way is presented in Table 9. Also exhibited in Table 9 are the fractions of the cell cycle occupied by the four stages G_1, S, G_2 and M calculated directly from the raw data, without adjustment for the fact that the cultures were in logarithmic growth when sampled, and also fractions adjusted so as to take account of this fact. The formulae necessary to make this adjustment were kindly provided by Professor R. Cormack, Department of Statistics, University of St. Andrews and are as follow:-

TABLE 9. Counts of *Xenopus* cells at various stages of the cell cycle made from Giemsa-stained autoradiographs of cultures pulse-labelled with ^{3}H-TdR.

Temperature of culture		G_1	S	G_2	M	Total
18°C	No. of cells	420	411	140	29	1000
	Fraction of T^a_g	0.42	0.41	0.14	0.03	
	Fraction of T^b_g	0.34	0.43	0.18	0.04	
25°C	No. of cells	1128	1200	313	26	2667
	Fraction of T^a_g	0.42	0.45	0.12	0.01	
	Fraction of T^b_g	0.34	0.48	0.15	0.01	
28°C	No. of cells	505	581	165	13	1264
	Fraction of T^a_g	0.40	0.46	0.13	0.01	
	Fraction of T^b_g	0.32	0.49	0.17	0.01	

T_g = generation time.

a = fraction of T_g (unadjusted).

b = fraction of T_g (adjusted).

If G_1, S, G_2 and M occur in the proportions w, x, y, and z, then the proportion of the cell cycle occupied by these phases are given as follow:-

$$G_1 = -\log_e \left(1-\frac{w}{2}\right)/\log^2 e,$$

$$S = -\log_e \left(1-\frac{x}{2-w}\right) \log^2 e,$$

$$G_2 = -\log_e \left(1-\frac{y}{2-w-x}\right)/\log^2 e,$$

and $$M = -\log_e \left(1-\frac{z}{2-w-x-y}\right)/\log^2 e$$

The adjusted fractions of the cell cycle occupied by G_1, S, G_2 and M have been inserted in Table 8 for comparison with the fractions obtained from the cell cycle analysis. They show reasonable agreement, though the agreement is still better if one makes comparison with the unadjusted fraction (see text-fig. 4)!

Fig. 5 shows the overall relationship between culture temperature, generation time, and the three portions of interphase. All are much more influenced by moving from 18°C to 23°C than from 23°C to 28°C, and all to approximately the same degree.

Text-fig. 4 shows the relationship between the cell cycle phases and the fraction of time spent in each phase as determined from:-

Cell cycle analysis (•————————→),

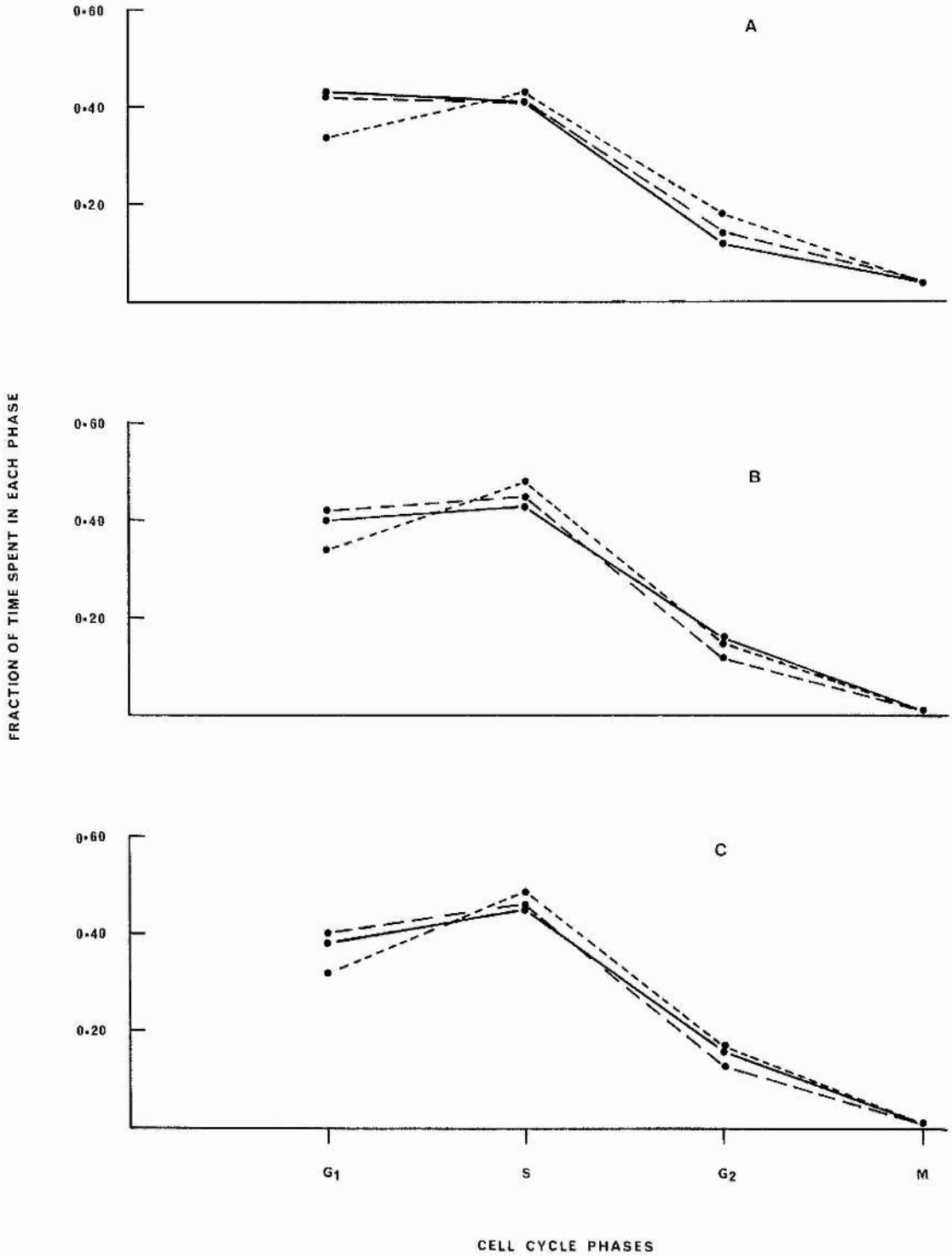
unadjusted cell counts (•— — — — — →)

and adjusted cell counts (•— — — — — — →).

A = cells grown at 18°C.

B = cells grown at 23°C.

C = cells grown at 28°C.


0·60
0·40
0·20
A
B
C
FRACTION OF TIME SPENT IN EACH PHASE
G1
S
G2
M
CELL CYCLE PHASES

Text-fig. 5 shows the variation in the durations of the cell cycle and its component phases according to growth temperature, obtained from pulse-labelled preparations of Xenopus cells in cultures at 18°, 23° and 28°C.

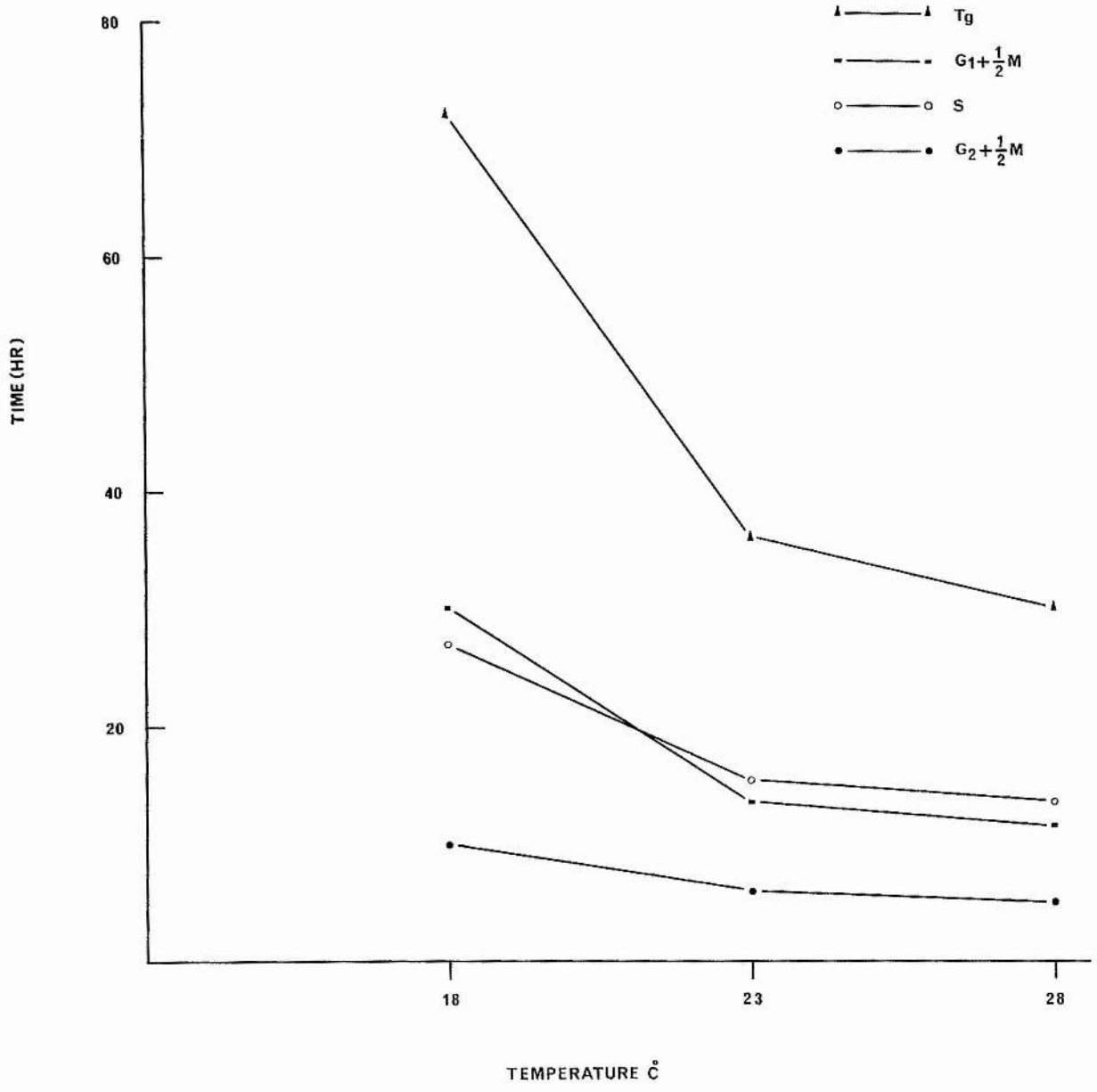
T_g = generation time.

G_1 = pre-synthetic period.

S = synthetic period.

G_2 = post-synthetic period.

M = mitoses.


80
60
40
20
TIME (HR)
Tg
G1+½M
S
G2+½M
18
23
28
TEMPERATURE C°

CHAPTER III.

Temperature and the distribution of sites where DNA synthesis is initiated

INTRODUCTION

It has by now become well recognised that the chromosomal DNA of eukaryotes replicates bidirectionally from many initiation sites which are tandemly arranged along the DNA double helices. This was first demonstrated by Huberman and Riggs (1968). They labelled Chinese hamster cells in tissue culture with a pulse of ^{3}H-TdR for a certain time and then replaced the medium with fresh medium this time lacking the radioactive-labelled precursor. In fibre autoradiographs they found many examples of dense grain tracks with "tails" of diminishing grain density at both ends. Huberman & Riggs explained this observation by assuming that DNA replication proceeds bidirectionally, the tails being the outcome of replication which occurred while cells used up their pools of precursors which had earlier been charged with ^{3}H-TdR from the labelled medium.

This method of labelling (pulse-chase) demonstrates not only the direction of DNA replication but also provides information about the distribution of initiation sites, the synchrony or otherwise of initiation, and fusions occuring between neighbouring origins.

Callan (1972) used the pulse-chase technique for labelling Xenopus somatic cells in culture at 25°C, and found that the distances between neighbouring initiation sites or initiation intervals range from 15 to 128 µm, with a mean of about 60 µm. He found also that, in Triturus cells, initiation intervals vary from one kind of cell to another in relation to

S-phase duration. The initiation intervals in Triturus embryonic cells are much shorter than those found in somatic cells, and these latter intervals are themselves much shorter than those present in spermatocytes during the very long drawn out premeiotic S-phase.

One of the aims of this study has been to see whether differences in culture temperature, which affects S-phase duration, affects the initiation intervals of Xenopus somatic cells in culture.

METHODS AND RESULTS

I followed the procedures of DNA autoradiography which were given in detail in Chapter (I) on "Materials and Methods" (page 14). Replicate subcultures of Xenopus cells were incubated for 48 hr at 28° and 23°C, and for 96 hr at 18°C. Thereafter the cells were treated with FUdR + UR for 20 hrs in the case of the 28° and 23°C cultures, or for 40 hrs in the case of the 18°C cultures, in order to synchronize most of them by preventing entry to the S-phase. Cells grown at 18°C were now provided with 50 µc/ml of ^{3}H-TdR (26 Ci/mM) for 4 hr, followed by a 4 hr stepdown period obtained by adding unlabelled thymidine (TdR) so as to reduce the activity of ^{3}H-TdR in the cultures medium to one quarter of the original. Cells grown at 23°C were similarly labelled at high specific activity for 2 hr, followed by a stepdown of 2 hr. Cells grown at 28°C were labelled for 1 hr, followed by a stepdown of 1 hr. After labelling the cells were trypsinized, harvested and dialysed as described in the Materials and Methods chapter1(page 16). The dialysis filters were covered with AR10 and exposed for 8 to 11 months at 4°C, developed and prepared for examination. The measurements were made directly from preparations using bright field illumination on a Zeiss microscope, with 12.5x eyepieces, one

containing a micrometer scale, optovar at 1.25, and 40x planapochromat oil immersion objective. This gave a magnification where 50 ocular divisions were equal to 100 μm.

Initiation intervals

Initiation sites or origins are places where DNA synthesis begins and initiation intervals are distances between neighbouring origins. In pulse-chase or pulse-stepdown labelling experiments, initiation intervals are most simply measured from midpoint to midpoint of adjacent dense stretches of silver grains where both dense stretches are flanked by tails, i.e. regions of diminished grain density, at either end. Autoradiographs of this kind are shown in figure 6, 12, 13 and 14. Where DNA synthesis had already initiated prior to the provision of ^{3}H-TdR, and continued after labelling, the resultant autoradiograph shows two dense stretches of silver grains with tails of diminished grain density towards the "outside", and a sharply demarcated gap between the dense stretches of silver grains towards the "inside". Such a gap represents the length of DNA replicated prior to labelling, and its midpoint may be taken as an origin. This gives further possibilities for measuring initiation intervals (see text-fig. 6 for further illustration). Examples are shown in figs 7, 8, 9, 10, 11 and 15.

Apart from one exceptional situation to be described later, initiation intervals can only be determined accurately from pulse-chase or pulse-stepdown labelling regimes, because only in such preparations can one identify direction of replication. This permits a clear distinction to be made between long labelled stretches which owe their length to fusion between neighbouring replication forks and those which owe their length to

Text-fig. 6 shows an illustration drawn to explain the procedures for measuring distances between adjacent origins (the initiation intervals) from preparations subjected to pulse/stepdown labelling regimes. A continuous thin line represents a length of parental DNA chain. Portions shown with large dots represent regions which replicated in the presence of label at high specific activity, portions shown with small dots represent regions which replicated during the stepdown period, and thin discontinuous lines represent regions which replicated before label had been introduced. O represents an origin or an initiation site.

I. - shows the arrangement of origins along a length of DNA chain.

II. - shows a pattern of labelled tracks where replication had begun before label was applied, and continued during the pulse and stepdown periods. O_1, O_2 and O_3 initiated replication at the same time.

III. - as II but O_1 and O_2 initiated replication before O_3. Fusion is assumed to have occurred between O_1 and O_2 during the stepdown period.

IV. - as II but fusions are assumed to have occurred between O_1, O_2 and O_3 during the stepdown period.

V. - as II but this time fusions are assumed to have occurred between O_1 and O_2 and between O_2 and O_3 before the stepdown period.

VI. - O_1 and then O_3 are assumed to have initiated replication before label was applied, while O_2 initiated during the presence of label. O_1, O_2 and O_3 continued replication during the pulse and stepdown periods.

VII. - as VI but fusion is assumed to have occurred between O_1 and O_2 during the stepdown period.

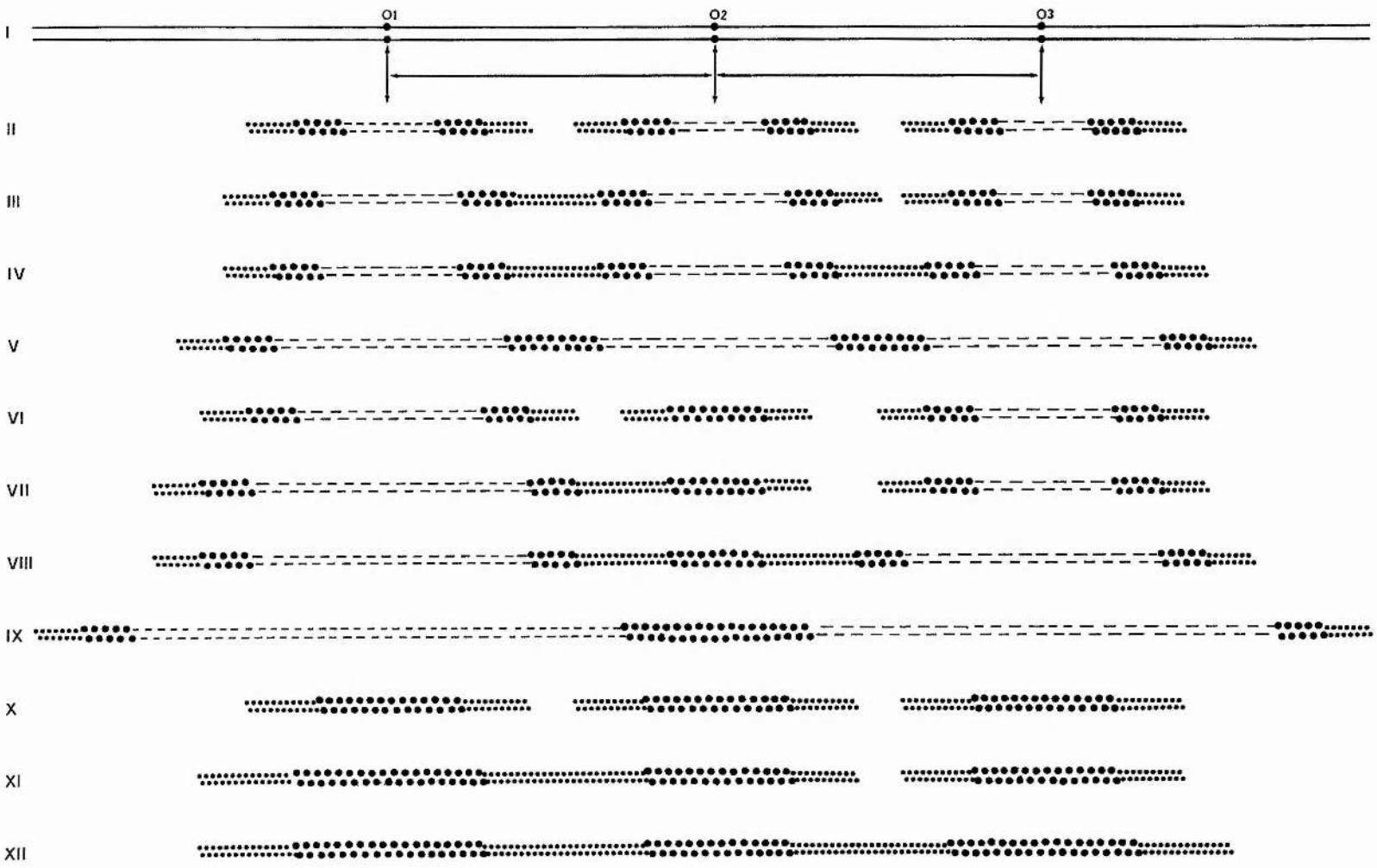
VIII. - as VI but this time fusions are assumed to have occurred between O_1 and O_2 and between O_2 and O_3 during the stepdown period.

IX. - as VI but fusions are assumed to have occurred between O_1 and O_2 and between O_2 and O_3, this time before the stepdown period.

X. - shows a pattern of labelled tracks where replication had initiated during the pulse and continued during the stepdown period. O_1, O_2 and O_3 are assumed to have initiated at the same time, and the increase in the length of these labelled tracks is assumed to be a consequence of extending the labelling periods.

XI. - as X, but O_1 is assumed to have initiated replication before O_2 and O_3, with fusion having occurred between O_1 and O_2 during the stepdown period.

XII. - as X, but this time O_1 and O_3 are assumed to have started replication before O_2, with fusions having occurred between O_1 and O_2 and between O_2 and O_3 during the stepdown period.


I
O1
O2
O3
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

replication having initiated when labelling started, and then proceeded throughout the labelling period. Examples are shown in the middle stretch of fig. 10, and the two replicating units of fig. 6.

The exceptional situation mentioned in the preceding paragraph is where, in preparations labelled without chase or stepdown, sister strand separation has occurred after neighbouring replication forks have fused and where neighbouring origins had already begun replication before label was provided. In favourable examples where the sister strands lie in register and one sees two identically interrupted stretches of silver grains running in parallel, the midpoints of the gaps between neighbouring grain tracks may be considered to be origins. In practice I found few examples of this situation, and made no use of these few examples for measuring intervals.

Text-fig. 7A shows the frequency distribution of 297 initiation intervals measured from autoradiographs of Xenopus kidney cells cultured at 18°C. The cells were pre-treated with FUdR + UR for 40 hr, labelled for 4 hr followed by a stepdown of 4 hr. The initiation intervals range from 26 to 136 μm about a mean of 65 μm.

Text-fig. 7B shows the frequency distribution of 475 initiation intervals measured from Xenopus cells cultured at 23°C. The cells were pre-treated with FUdR + UR for 20 hr, labelled for 2 hr followed by a stepdown of 2 hr. The initiation intervals here range from 10 to 144 μm, again about a mean of 66 μm.

Text-fig. 7C shows the frequency distribution of initiation intervals measured from cells in culture at 28°C. These cells were pre-treated with FUdR + UR for 20 hr and labelled either for 1 hr only, or for 1 hr with a stepdown of a further 1 hr.

Text-fig. 7A shows the frequency distribution of 297 initiation intervals measured from autoradiographs of Xenopus kidney cells cultured at 18°C. The cells were pre-treated with FUdR + UR for 40 hr, labelled for 4 hr followed by a stepdown of 4 hr. The initiation intervals range from 26 to 136 μm about a mean of 65 μm.

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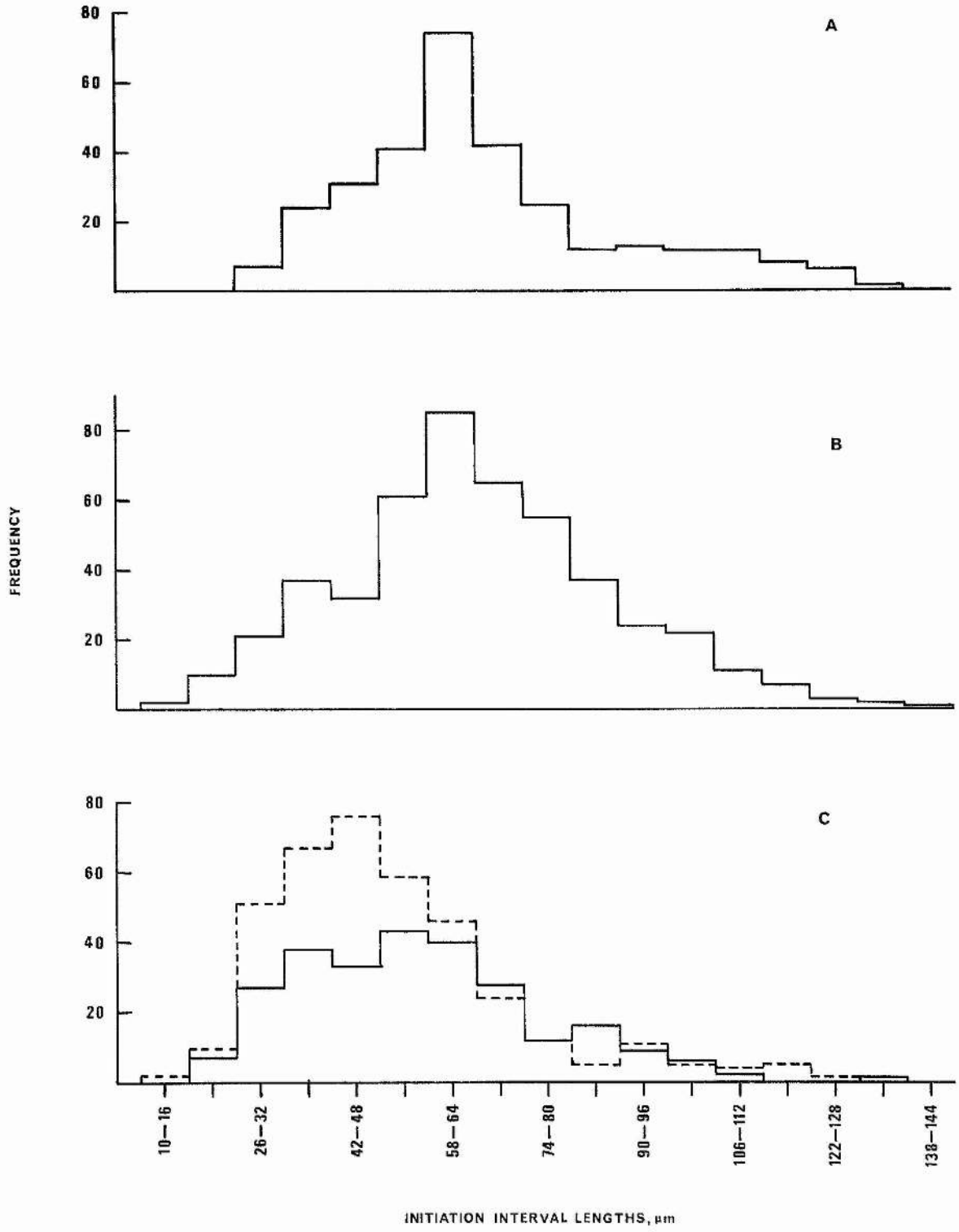
Text fig. 7C shows the frequency distribution of initiation intervals measured from cells in culture at 28°C. These cells were pre-treated with FUdR + UR for 20 hr and labelled either for 1 hr only (— — — —) giving a mean interval of 50 μm or for 1 hr with a stepdown of a further 1 hr (————) giving a mean interval of 55 μm.


A
B
C
80
60
40
20
FREQUENCY
10—16
26—32
42—48
58—64
74—80
90—96
106—112
122—128
138—144
INITIATION INTERVAL LENGTHS, μm

The 28°C stepdown preparations were of poor quality, and for this I have no ready explanation. Although they were exposed for 8 to 12 months, the grain tracks were weak, and on only one slide were they sufficiently well defined to measure. This slide yielded 260 intervals, which are shown as a histogram in text-fig. 7C. The intervals range from 18 to 136 μm about a mean of 55 μm.

I attempted to score initiation intervals from several preparations of the 28°C cells where the labelling was not succeeded by a stepdown, knowing well the likely errors of so doing. If neighbouring initiations were synchronous and replication occurred when the FUdR block was relieved by the provision of labelled thymidine, then assuming that all DNA is bidirectional from origins one could take the midpoints of labelled tracks to be origins. In practice, however, we know that neighbouring initiations are not synchronous, and moreover a proportion of the tracks seen will be registering one-way replication from origins which had already initiated before labelled thymidine was provided. Furthermore some tracks will necessarily be registering fusion between converging replication forks proceeding before the provision of label. Unable to discriminate between these various possibilities, the best one can do is simply to measure midpoint to midpoint intervals between all tandem tracks, knowing that this will tend to underestimate initiation intervals because of inclusion of tracks registering one-way replication. 381 intervals were scored from 1 hr labelled preparations, and these are shown as a histogram in text-fig. 7C, alongside the histogram derived from the single and rather unreliable stepdown slide. The intervals range from 10 to 120 μm about a mean of 50 μm and as anticipated tend to be somewhat shorter than the intervals derived from the stepdown slide.

Callan (1972) has measured the intiation intervals for this cell-line in culture at 25°C. He found that the intervals range from 18 to 128 μm about a mean of 60 μm. Comparing the results of Callan at 25°C with mine at 18° and 23°C, it seems reasonable to assume that the mean initiation interval at 28°C is also around 60 μm, and if this is so then the initiation intervals are evidently not influenced by culture temperature despite the influence of temperature on S-phase duration.

The direction of DNA replication

It has been generally accepted that pulse-chase or pulse-stepdown labelling provides direct evidence as to the way DNA synthesis proceeds. DNA fibre autoradiographs of cells which have been subjected to two successive pulses of ^{3}H-TdR, and first high and the second low in their specific activities, show dense grain tracks (where replication started during the first pulse) flanked by tails of declining grain density as a result of dilution of the specific activity during the stepdown. If replication were to proceed in one direction, the dense labelled tracks would show tails at one end, and if the replication is bidirectional the tracks should show tails at both ends.

The results of this study fully substantiate the view that the replication of the chromosomal DNA of Xenopus proceeds bidirectionally (figs. 6, 12, 13 and 14). The majority of the origins which one sees had initiated only after the FUdR block had been removed, and these show the expected tails at both ends. There are, however, a minority of origins, coming from cells which were already in S when the FUdR block was applied, where replication was already in progress prior to the provision of ^{3}H-TdR. These continue to replicate in the presence of ^{3}H-TdR, and provided

fusion between neighbours does not occur during the pulse period, they show up in autoradiographs as dense tracks with a tail at one end only. Far more frequently than chance would allow, one sees such tracks with single tails "back-to-back", i.e. with both tails directed towards the outside (figs. 8,9, 10, 11). Such tracks equally confirm that replication proceeds bidirectionally from origins.

Further evidence that replication occurs bidirectionally from origins comes from tracks which have registered fusion, or better still near fusion, between adjacent units during the stepdown (figs. 6, 10 & 13). These regularly show fusion occurring between "tails", towards the outside of which are dense tracks. The point to emphasize is that the fusions are tail to tail, and not between the tail of one track and the dense region of an adjacent track. If the latter were found, this would indicate that at least one of the fusing tracks had originated from an initiation site from which replication had proceeded in one direction only; that they are not found is strong evidence against the notion that any origins initiate replication in one direction only.

Fig. 6 is a particularly clear example, in which fusion had evidently occurred just at the time of harvesting. One can infer the position of initiation site 1, while initiation site 2 is marked by slight separation of the sister duplexes. The tails are of symmetrical length about these two origins, so the less dense track which includes the point of fusion must surely represent the fusion of two tails, not the fusion of one exaggeratedly long tail coming from, say origin 1, and the dense track coming from origin 2.

Sister strand separation, asynchrony of initiation times, and termini

Pulse-stepdown labelled DNA fibre autoradiographs show that newly replicated strands start to separate a certain time after the initiation of the replication. For example, sister strand separations are apparent within labelled tracks only after 8 hr of labelling at 18°C (fig. 25) and after 4 hr at 28°C (figs. 48, 50 & 58). Pulse-stepdown preparations show not only that sister strand separation occurs after a certain time (figs. 6 and 15) but also give a clear idea concerning the relative times of initiation of neighbouring origins and whether or not termini exist. Figs. 7, 8, 9 and 10 show tracks where replication had already initiated before labelling and continued during the 8 hr of labelling (4 hr high + 4 hr low at 18°C), with unlabelled gaps bounded by heavily labelled tracks with tails to the outside. The arrows indicate the location of initiation sites, which are assumed to be at the middle of each gap. Sites number 1 and 2 of fig. 10 appear as though they had initiated at the same time because the lengths of the two unlabelled gaps are the same. Site number 2 of fig. 9, on the other hand, had evidently initiated slightly before site 1. Site number 3 of fig.8 had initiated a little before 2, but the adjacent site 1 only initiated some time after applying the ^{3}H-TdR; there is no gap in this labelled track and the track is shorter than its neighbours. A particularly good example of staggering in the activation of initiation sites can be seen in fig. 15. This photograph was taken from a DNA fibre autoradiograph of Xenopus cells in culture at 23°C. The cells were labelled with ^{3}H-TdR for 2 hr followed by a stepdown of 2 hr. It shows that site number 1 had initiated before sites 2 and 3, and that site 3 had initiated before 2. Fig. 15 also shows a good example of sister strand separation, bidirectional replication

and fusions which had taken place between replicating tracks proceeding from sites 1 and 2 and between 2 and 3 during the first 2 hr labelling period, i.e. before the stepdown (see the illustration in text-fig. 8).

Termini are assumed to be points where a replication fork waits until a neighbouring replicating fork comes into its proximity, and there has been some discussion as to whether or not they exist (Huberman & Riggs, 1968; McFarlane & Callan, 1973). If termini exist, then DNA fibre autoradiographs from cells subjected to stepdown labelling might be expected to show, at least occasionally, dense labelled tracks where one has a tail and the other none, or at least evident asymmetry. One of the very few examples of a track showing evidence for a terminus is to be seen in fig. 8, the track above initiation site 2, which is decidedly shorter than its partner below, the latter having a defined tail. I would stress the fact that this is exceptional, and that such tracks as are shown in figure 15 provide strong evidence against the existence of termini symmetrically spaced on either side of an origin, which was Huberman & Riggs (1968) early claim.

Generally my results argue against the existence of termini; a replication fork seems to proceed without interruption until it meets a neighbouring fork coming from the opposite direction.

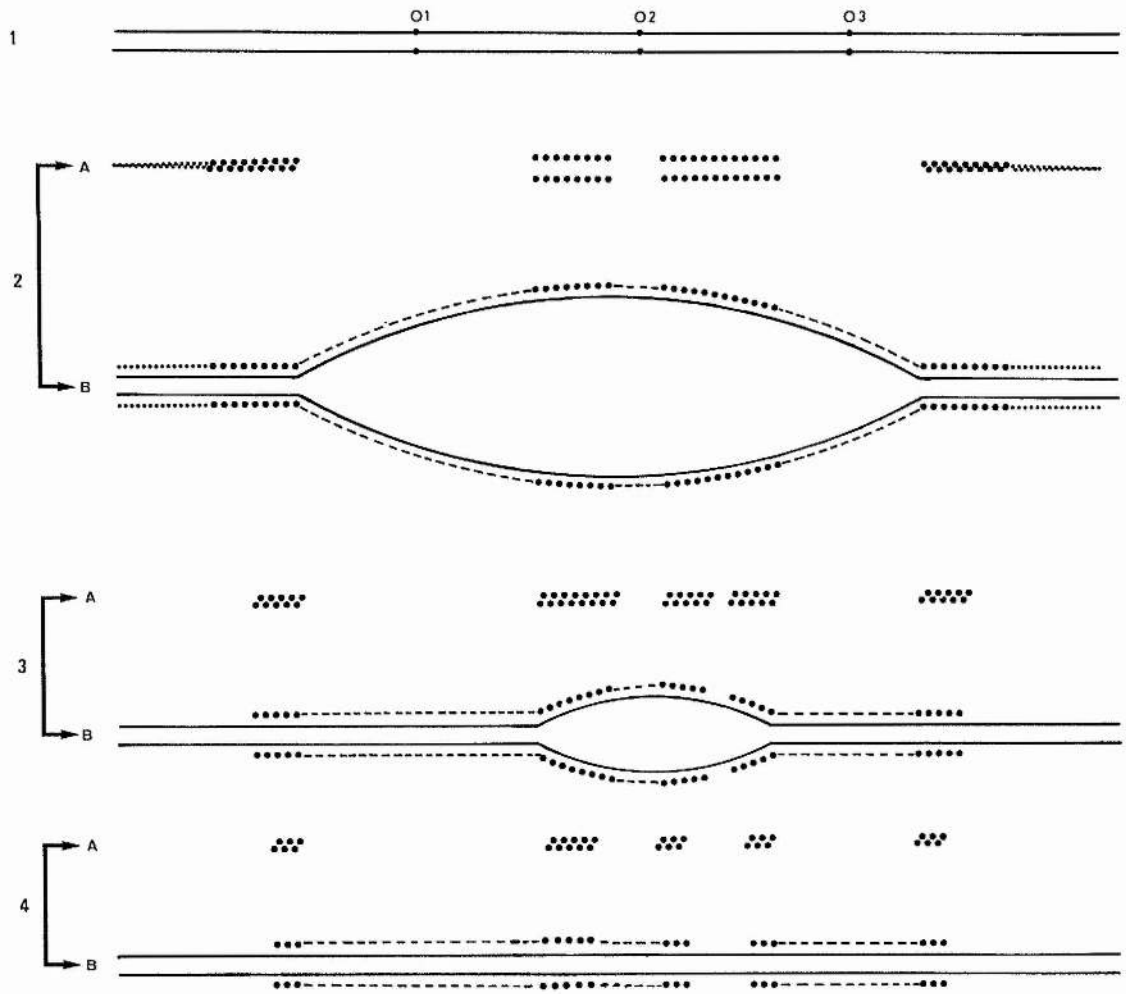
Text-fig. 8. An illustration drawn to show details of replication along a length of chromosomal DNA. Continuous lines represent parental polynucleotide chains. Thin broken lines represent polynucleotide chains synthesized before labelling. Lines of large dots represent polynucleotide chains synthesized during labelling at high specific activity. Lines of smaller dots represent polynucleotide chains synthesized during labelling at lower specific activity. Drawings labelled A signify tracks which have been observed, or might be observed, in fibre autoradiographs, while drawings labelled B are the relevant interpretations.

1. shows the parental DNA with initiation sites of O_1, O_2 and O_3.

2. shows tracks as those illustrated in fig. 15 (2 hr pulse 2 hr stepdown, at 23°C),

3. shows tracks which would have been observed if the DNA fibre replicating as in fig. 2 had instead been harvested after 2 hrs of labelling only.

4. as in fig. 3, but the result of harvesting at 1 hr of labelling only.


1
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2
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B
3
A
B
4
A
B

CHAPTER IV.

Temperature and the rate of progress of replication forks

INTRODUCTION

Several studies on mammalian cells in culture have demonstrated that the durations of the various parts of the cell cycle are temperature-dependent (Rao & Engelberg, 1965; Sisken *et al.*, 1965; Watanabe & Okada, 1967; Saladino & Johnson, 1974). Replication of DNA occurs during a part of the cell cycle, the S-phase, and as this is one of the phases dependent on temperature for its duration there clearly is some relationship between the overall rate of DNA synthesis, and temperature. Mammalian cells in culture do not tolerate a considerable temperature range, whereas the *Xenopus* A-6 cell line can grow satisfactorily anywhere within the range 18° to 28°C.

This chapter will be concerned with the determination of the rates of progress of replication forks in *Xenopus* cells cultured at 18°C, 23°C, and 28°C.

METHODS AND RESULTS

The details of subculturing, treatment with FUdR and labelling are given in table 10. Cells grown at 18°C were allowed to grow for 96 hrs so as to enter the log phase, were then blocked for 40 hrs with FUdR to accumulate cells ready to enter the S-phase, and were then labelled with ^{3}H-TdR at high specific activity for 2, 4 or 8 hrs prior to harvesting. Cells grown at 23°C were maintained for 48 hrs prior to an FUdR block of 20 hrs, then labelled for 2 or 4 hrs. Cells grown at 28°C were similarly maintained for 48 hrs and blocked for 20 hrs, then labelled for 1, 2, or 4 hrs.

TABLE 10. Schedules of experimental proocdures.

Temperature °C	Times prior to FUdR treatment hrs	Durations of FUdR treatment hrs	Periods of labelling hrs
18	96	40	2 4 8
23	48	20	2 4
28	48	20	1 2 4

20 hrs was chosen for the FUdR block applied to the 23°C and 28°C cultures because the use of a block of this duration for cells cultured at 25°C is known to produce roughly 50% synchronization of cells entering S when the block is relieved (Callan, 1972). The cell cycle time at 28°C is 30 hrs, and at 23°C is 36 hrs, whereas at 18°C it is considerably longer, 72 hrs. Thus for the cells cultured at 18°C the duration of the FUdR block was deliberately doubled.

After labelling the cells were harvested, and DNA fibre autoradiographs prepared according to the methods already described in Chapter I. Silver grain track lengths were measured directly from the preparations using an eye-piece micrometer and magnification such that 50 micrometer divisions represent 100 μm.

Track lengths chosen for measurement were selected from regions of the autoradiographs where fibre density was low, and where a tandem arrangement of 2, 3 or more tracks could be clearly recognised. By selecting tracks from within sparsely labelled regions one can avoid making such observational errors as confusing 2 separate but partly overlapping tracks for a single track. Moreover by selecting straight tandemly arranged tracks for measurement one can be reasonably sure that the DNA fibre had been well stretched and unbroken on the Millipore filter prior to the application of AR10.

Track length measurements from cells cultured at 18°C

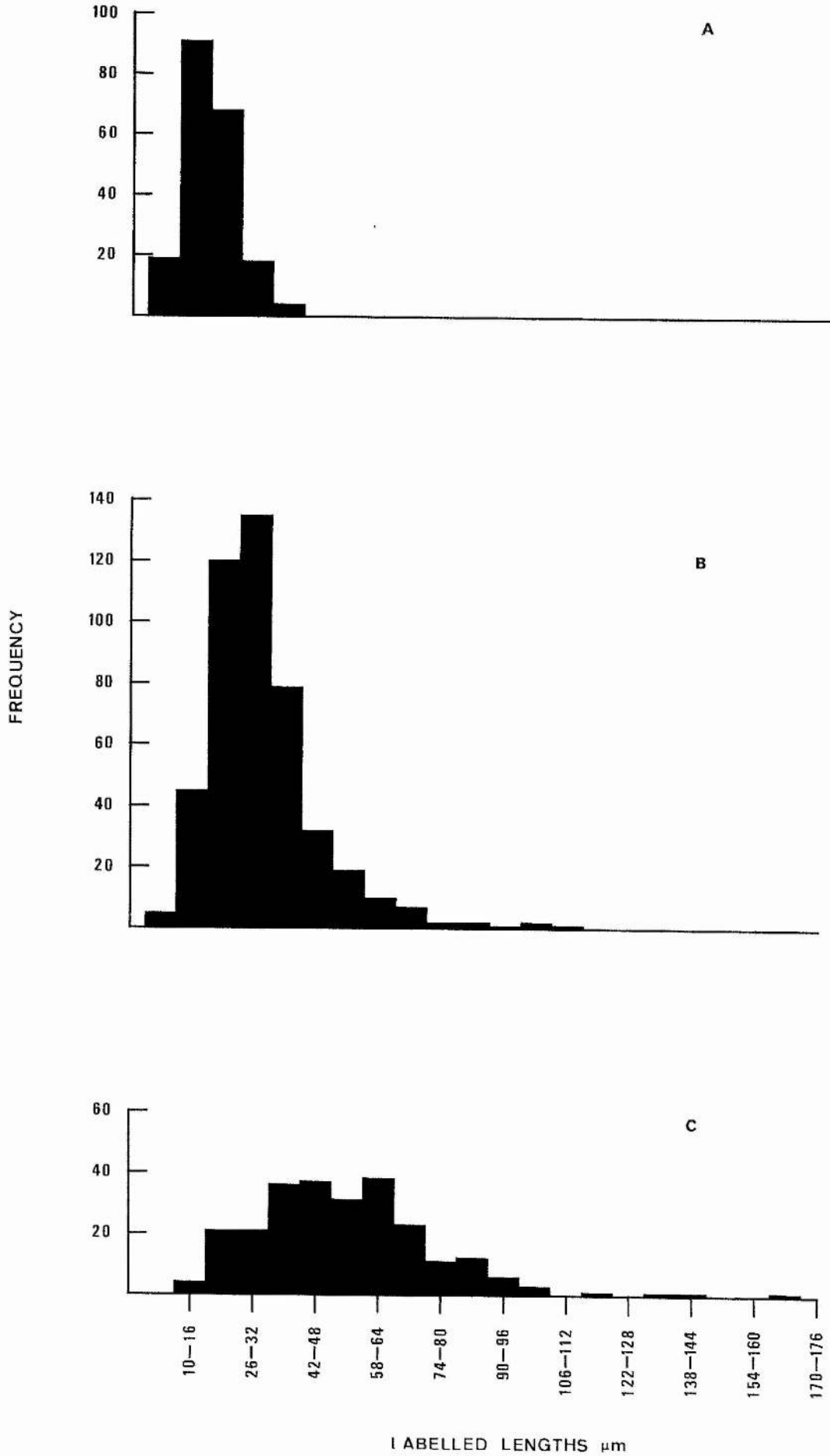
Typical tracks from cultures labelled for 2 hours are shown in figures 16-18. The frequency distribution of the lengths of 200 such tracks is shown as a histogram in text-fig. 9A. Typical tracks after 4 hrs of labelling are shown in figs 19-21 and the frequency distribution of the lengths of 460 such tracks is shown in text-fig. 9B. Typical tracks after 8 hrs of labelling are shown in figs 22-27, and the corresponding histogram derived from 247 measured tracks is shown in text-fig. 9C.

Autoradiographs such as these include tracks which register where DNA synthesis had initiated at the beginning of labelling, and had proceeded bidirectionally until the time of harvesting, with or without fusions to neighbouring units. Other tracks register bidirectional replication from later-starting origins, while yet others register one-way replication, all of these with or without fusions during the period of labelling. These various situations are illustrated in text-fig. 10.

In order to make a realistic estimate of the rate of progress of replication forks one can choose a period of labelling so restricted that few or no fusions have occurred between any of those neighbouring units which initiated during the labelling period. The longest tracks recorded in preparations made after such a short labelling period should give a measure of the 2-way rate, always provided that no fused tracks have been included among the observations.

Histogram 9A (2 hrs of labelling) shows a steep rise and an equally steep fall in the 18 - 24 μm track range, with relatively few (20) tracks longer than this. These latter may perhaps be accounted for by occasional fusions between replicating units converging from particularly close

Text-fig. 9 (A, B & C) are histograms of the frequency distributions of track lengths as measured from DNA fibre autoradiographs prepared from Xenopus cells cultured at 18°C. The cells were treated with FUdR for 40 hrs and then labelled with ^{3}H-TdR for 2 hrs (A), 4 hrs (B) and 8 hrs (C).


A
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FREQUENCY
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120
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60
40
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10—16
26—32
42—48
58—64
74—80
90—96
106—112
122—128
138—144
154—160
170—176
LABELLED LENGTHS μm

Text-fig. 10 shows an interpretative drawing of varieties of labelled tracks which might be seen within DNA fibre autoradiographic preparations. A continuous line represents a portion of parent duplex DNA, a dotted line represents new labelled DNA strands which initiated replication during the labelling period, a broken line represents new DNA strands which initiated replication before the labelling period and O represents initiation origins or sites. A are patterns of labelled tracks as might be seen in autoradiographic preparations and B are illustrations of the arrangement of labelled strands alongside the parental strands.

1 - shows two strands of parent duplex DNA and the location of initiation sites or origins along them.

2 - shows four labelled tracks where replication initiated at the beginning of the labelling period.

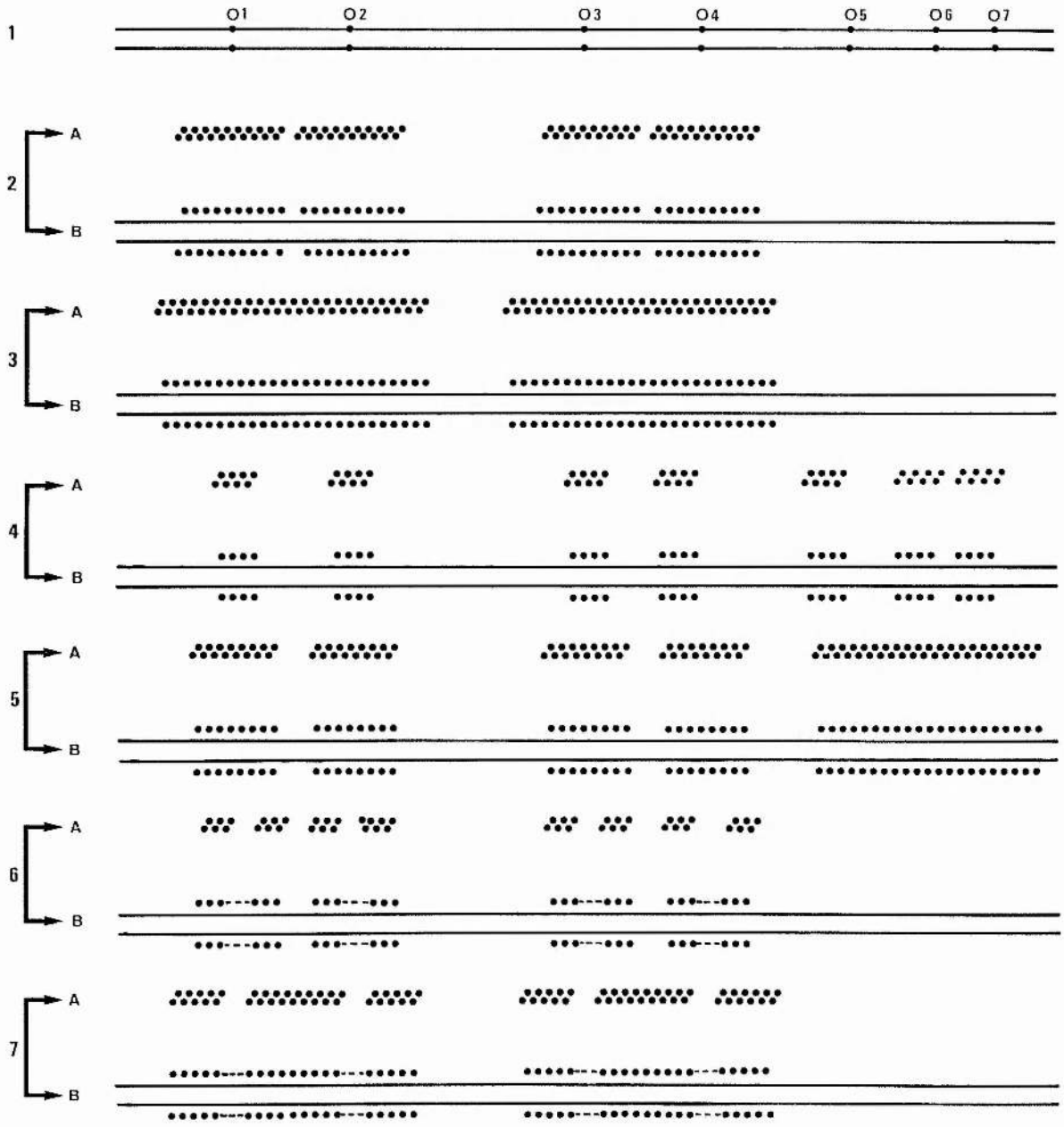
3 - as 2 but fusions took place between units 1 and 2 and between units 3 and 4.

4 - shows seven labelled tracks where replication initiates some time after the beginning of the labelling period.

5 - as 4 but fusions took place between units 5 and 6 and 7.

6 - shows four labelled tracks where replication had initiated before the labelling period, and continued in the presence of label.

7 - as 6 but fusions took place between units 1 and 2 and between units 3 and 4.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4
A
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A
B
6
A
B
7
A
B

origins, or they may be a consequence of a replication fork rate that is higher than the normal. However they be explained, I would judge that they can be ignored, and that one should take the position of the steeply falling right hand shoulder (RHS) of the histogram as a realistic measure of 2-way replication which has progressed in 2 hrs. This is 24 μm, giving a one-way rate of 6 μm/hr. It is manifestly not valid to use the mean track length of this histogram (17 μm) as a measure of the 2-way rate, because the histogram necessarily includes some one-way tracks, and also 2-way tracks which initiated late.

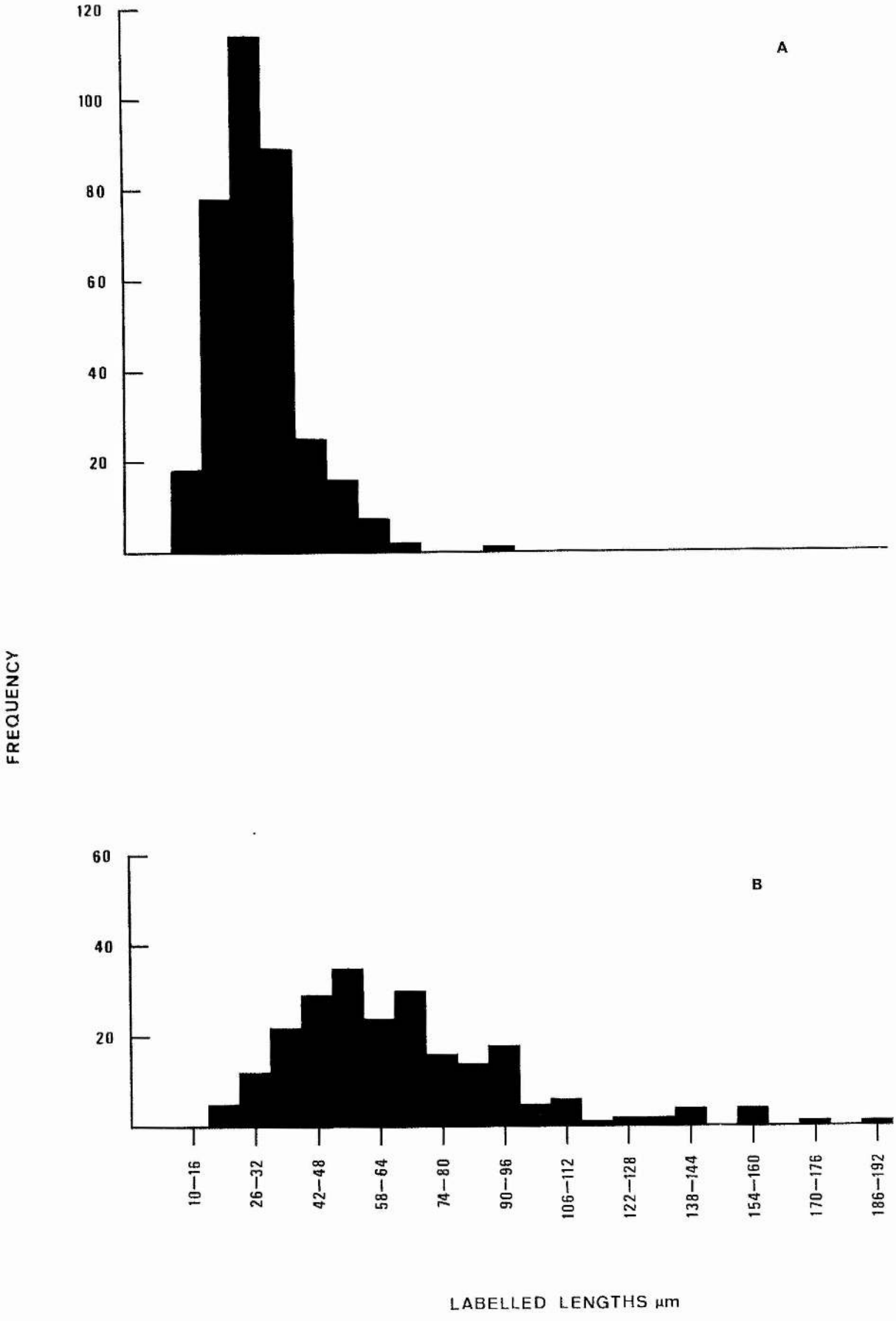
Histogram 9B (4 hrs of labelling) is very nearly as "peaked" as 9A. Again, if one discounts the right hand tail, the RHS at 40 μm gives a one-way rate of 5 μm/hr. This is in reasonable accord with the rate estimated from the 2 hr preparations.

Histogram 9C (8 hrs of labelling) is very flat by contrast with 9A and 9B. One cannot recognize a well defined RHS, and a glance at figures 24 and 25 show that fused tracks must have been extensively represented in the measurements. This is borne out by the evidence of the 4 hr + 4 hr stepdown autoradiographs shown in figures 6 and 10.

Track length measurements from cells cultured at 23°C

Typical tracks from cultures labelled for 2 hrs are shown in figures 28-32; and the frequency distribution of the lengths of 350 tracks is shown as a histogram in text-figure 11A. Tracks after 4 hrs of labelling are shown in figures 33-40; and the frequency distribution of the lengths of 229 tracks is shown in text-fig. 11B.

Text-fig. 11 (A & B) are histograms of the frequency distributions of track lengths as measured from DNA fibre autoradiographs prepared from Xenopus cells cultured at 23°C. The cells were treated with FUdR for 20 hrs and then labelled with ^{3}H-TdR for 2 hrs (A) and 4 hrs (B).


A
120
100
80
60
40
20
B
60
40
20
FREQUENCY
10—16
26—32
42—48
58—64
74—80
90—96
106—112
122—128
138—144
154—160
170—176
186—192
LABELLED LENGTHS µm

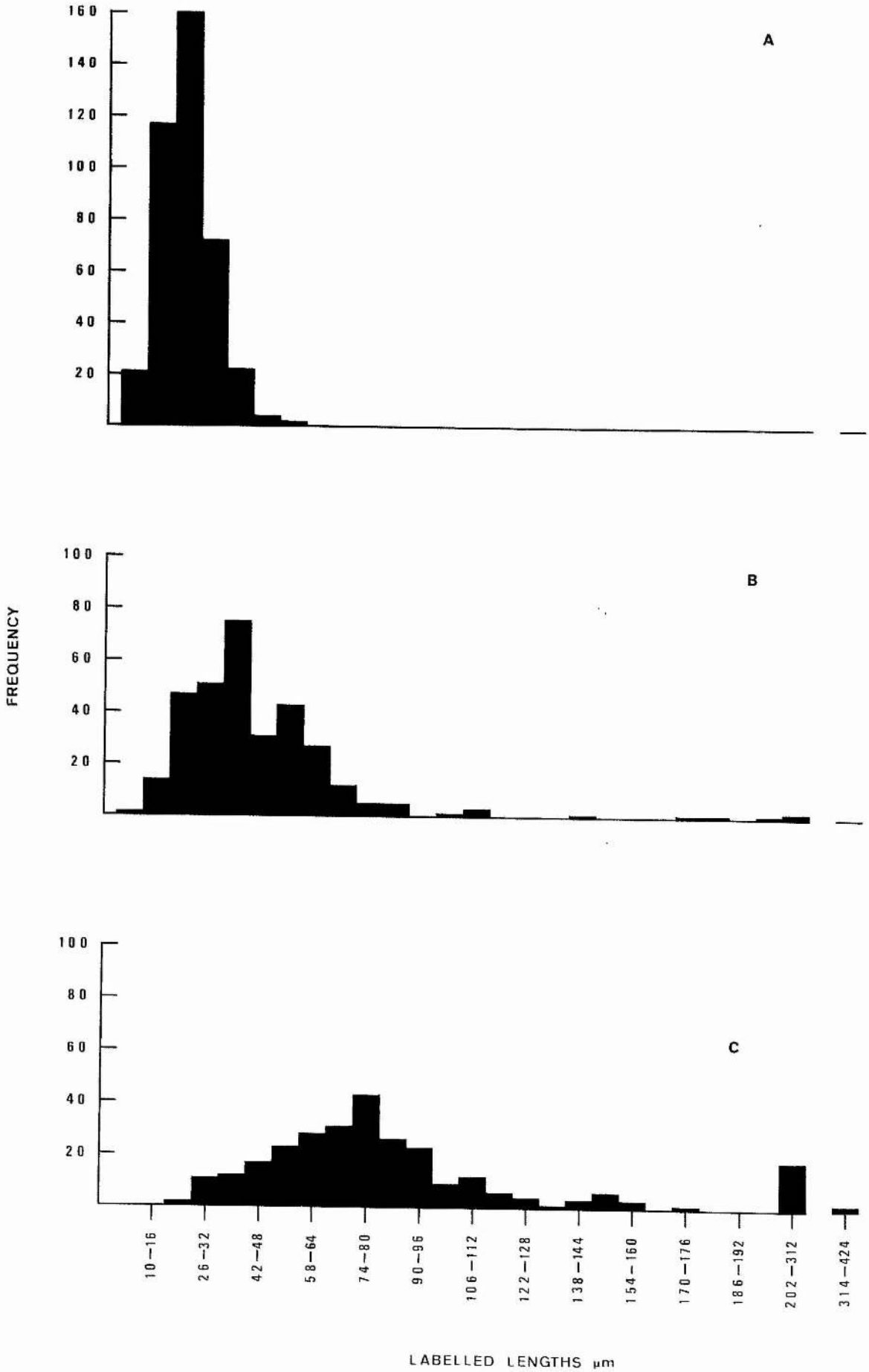
The 2 hr histogram 11A shows that the lengths of labelled tracks range from 10 to 96 μm with a mean length of about 32 μm. Choosing the RHS rather than the mean (for the reasons previously mentioned) for estimating the value of the replication rate, this would appear to be about 40 μm, i.e. the one-way replication rate at this temperature is about 10 μm/hr. In the 4 hr histogram the range of labelled track lengths is between 18 and 192 μm, but one cannot recognise a defined RHS on this flat histogram evidently because of many track fusions occurring within this labelling period. This is in no way surprising; 4 hours of replication at 10 μm/hr one-way will replicate 40 μm of DNA from a single origin, and converging from a neighbouring origin, 40 μm likewise, i.e. a total of 80 μm; but 70% of the measured initiation intervals are 80 μm or less.

Reference to text-fig. 7B shows that out of 475 initiation intervals measured from 2 hr pulse/2 hr stepdown preparations, 368 are 80 μm or less, and these would be expected to fuse within 4 hours. However because two adjacent replicating units which fail to fuse produce two tracks, whereas two adjacent units which do fuse produce only one, (Callan, 1972) then by calculation the proportion of fused to total tracks should not be 368 out of 475 (70%) but rather 368 out of 582 (368 + 2 x 107), or 63%.

Track length measurements from cells cultured at 28°C.

Typical tracks from cultures labelled for 1 hr are shown in figs 41 to 43 and the frequency distribution of the lengths of 400 tracks is shown as a histogram in text-figure 12A. Tracks after 2 hrs of labelling are shown in figs 44 to 46, and the frequency distribution of the lengths of 315 tracks is shown in text-fig. 12B. Tracks after 4 hrs of labelling are shown in figs 47 to 52, and the frequency distribution of the lengths of 254 tracks is shown in text-fig. 12C.

Text-fig. 12 (A, B & C) are histograms of the frequency distributions of track lengths as measured from DNA fibre autoradiographs prepared from Xenopus cells cultured at 28°C. The cells were treated with FUdR for 20 hrs and then labelled with ^{3}H-TdR for 1 hr (A), 2 hrs (B) and 4 hrs (C).


A
B
C
FREQUENCY
160
140
120
100
80
60
40
20
100
80
60
40
20
100
80
60
40
20
10–16
26–32
42–48
58–64
74–80
90–96
106–112
122–128
138–144
154–160
170–176
186–192
202–312
314–424
LABELLED LENGTHS µm

Histogram A (1 hr labelling) is well peaked, and the RHS falls at about 32 µm. This gives an estimate for the one-way replication rate of 16 µm/hr. Histogram 13B (2 hr labelling) does not have as sharp a RHS as 12A; late initiations tend to drag the histogram to the left, while fusions produce a tail to the right. However one has the impression of a steep decline somewhere in the region of track lengths 50 to 64 µm. If one takes as a working figure 58 µm, the replication rate calculates as 14.5 µm/hr. Longer labelling times inevitably tend to underestimate replication rate, but somewhere in the neighbourhood of 15 µm/hr is probably a fair estimate, bearing all sources of inaccuracy in mind.

Histogram 12C evidently includes many fusions, in accord with the photographs shown in figs 47, 48, 52, and 58. Considering the rate at 28°C to be about 15 µm/hr one-way, 4 hr of replication will replicate 60 µm, and again converging from a neighbouring origin, 60 µm likewise, i.e. a total of 120 µm. The initiation intervals which are measured from 1 hr pulse 1 hr stepdown preparations (text-fig. 7C) include scarcely any intervals in excess of 120 µm, so a very high frequency of fusion is to be expected.

GENERAL DISCUSSION

Mammals and birds have constant body temperatures and mammalian cells in culture grow best at 37°C. Below or above this optimum temperature cell growth is impaired (see Table 11). The metabolic enzymes in mammals and birds have optimal temperatures in the range of 30° to 40°C at which they are relatively stable (Hardy, 1972). Although Amphibia do not regulate their body temperature, it is likely that amphibian cells have growth temperature optima in tissue culture. Seto & Rounds (1968) mention that frog kidney cells (Rana nigromaculata) and newt lung cells (Taricha torosa) in tissue culture grow best at 26°C, are retarded below 22°C, and inhibited at 37°C. But as pointed out (page 25) it cannot be stated with assurance that Xenopus cells in culture do have an optimum temperature; the argument which should be emphasized is that temperature influences the relative durations of the stages of the cell cycle. Chibon (1973) found that the cell cycles of Pleurodeles waltlii embryonic cells are temperature-dependent, and that the synthetic period decreases sharply between 12°C to 17°C but less so above 17°C. In the experiments described here using Xenopus cells, a sharp decrease in the durations of G_1, S, G_2 and M is observed over a temperature increase from 18° to 23°C, whereas from 23° to 28°C only a slight decrease in durations of the cell cycle stages is observed. The increasing durations of the cell cycle as temperatures are reduced must in part be ascribed to the general property of chemical reactions that their rates are directly dependent on temperature. However what must also be taken into account are the temperature optima of the various enzymes involved in cell "housekeeping" functions such as the synthesis of DNA, RNA and proteins, and their precursors. The disproportionate increase in mitotic time at 18°C may well be due to an inhibition of spindle assembly (see Barber & Callan, 1943). Rao & Engelberg

TABLE 11. The effect of varying temperature on generation times (Tg) and synthetic period (S) of mammalian and amphibian cells in tissue culture.

Temperature	Mammals				Amphibia			
°C	HeLa cells		Human amnion		Pleurodeles embryo, stage 34 Fore-limb mesenchyme		Xenopus kidney cells (A-6)	
	Tg (hr)	S (hr)	Tg (hr)	S (hr)	Tg (hr)	S (hr)	Tg (hr)	S (hr)
12					91	79		
17					40	31		
18							72	29.5
23					24	16	36	15.5
26					22	14		
28							30	13.5
34	44.8	14.8	30.5	10.3				
36	25.8	7.4	21.8	8.3				
37	21.8	7.0	19.5	7.0				
40	33.7	11.2	25.3	9.1				
	Rao & Engelberg 1966		Sisken, et al., 1965		Chibon 1973		The results of this study	

(1966) have similarly shown that the increase in the duration of mitosis in mammalian cells (HeLa cells) at low temperature is disproportionately greater than that of the other phases of the cell cycle.

The lengthening of G_2 as a consequence of low temperature may be due to a reduced rate of non-histone protein synthesis, including those proteins that are necessary for chromosomal condensation (Anderson, 1956; Swift, 1964; 1965; Frenster, 1965). Several studies indicate that such proteins associated with mitotic chromosomes are synthesized continuously throughout interphase (Prescott & Bender, 1963; Prensky & Smith, 1964; Cherick & Davidson, 1968; Gerner & Humphrey, 1973). Similarly, the lengthening of the G_1 period could be due to a reduction in the rate of synthesis of high molecular weight proteins (above 45 KD); these latter are known to be synthesized at a particularly high rate in the late G_2 (Gerner & Humphrey, 1973).

Pulse labelling and subsequent Giemsa staining of tissue culture cells enables a quick and convenient estimate to be made of the duration of the cell cycle stages. Immediately, it can be seen that nuclei in S-phase are labelled, nuclei in G_1 are small and unlabelled and nuclei in G_2 are large and unlabelled. Lyndon (1973) has shown that it is possible to calculate the proportions of 2C and 4C nuclei in the shoot apex of Pisum by measuring the diameters of nuclei; he found significant differences between nuclear diameters of 2C and 4C nuclei.

Cell cycle examinations, although informative as to the effects of treatments on the stages of the cycle, shed little light on the causality. In order to discriminate between the metabolic effects of temperature change or physical effects, such as are perhaps manifested in DNA/histone packing, it is necessary to perform experiments where analysis can be made

at the molecular level. The effects of temperature on the duration of the S-period may be due to altered rates of DNA synthesis, which may in turn have several controlling factors. DNA fibre autoradiography helps to resolve some of these factors.

Pulse-stepdown preparations indicate that the mean initiation intervals (or the distances between adjacent active initiation sites) in Xenopus cells in culture do not vary significantly with temperature. The mean initiation intervals for cells cultured at 18°C and 23°C is the same, in the order of 66 µm. Cells cultured at 28°C have a mean of about 50 to 55 µm but the data for 28°C comes from rather poor and unsatisfactory preparations. Callan (1972) has found that the mean initiation intervals for Xenopus cells in culture at 25°C is about 60 µm. Callan's findings have encouraged me to assume that the mean distance between adjacent active sites is about 60 µm at 28°C.

The S-phase durations at 18°, 23°, and 28°C are 29.5 hr, 15.5 hr and 13.5 hrs respectively. If the initiation intervals are the same at all three temperatures, then either varying rates of fork progression, or varying degrees of staggering of the activation of origins, or both together, must be responsible for the temperature dependence of S-phase duration.

Table 12 shows the results of several studies which demonstrate that initiation intervals vary between cell types. The most interesting results, and those most relevant to the results described here, are Callan's findings on amphibian cells (1972; 1973; 1976). Callan showed that Triturus embryonic cells have much shorter S-phases and also much smaller initiation intervals than have somatic cells. Pre-meiotic spermatocytes, on the other hand, have an extremely long S-phase with correspondingly long initiation intervals. The ultimate S-phase for Triturus spermatocytes is about 200 hrs (Callan &

TABLE 12. A comparison of data concerning S-phase duration, replication rate and initiation intervals at different temperatures.

Temperature °C	S-phase duration hr	Replication rate one-way µm/hr	Range of initiation intervals µm	Mean of initiation intervals µm	Cell type	Ref.
37	9-10	36	40-280	130	Human (culture)	1
37	6-8	30-72	15-120	50	Chinese hamster (culture)	2
37	7.5	25-30	25-145	63	Gallus domesticus (culture)	3
25	13	9	18-128	60	Xenopus laevis somatic cells (culture)	4
25	48	20	100-350+	?	Triturus cristatus somatic cells (culture)	4
18	200	12	$\gg$100	?	Triturus vulgaris spermatocytes	4
18	4-6	6	40		Triturus vulgaris neurula	5
18	1	3	$<$10		Triturus vulgaris blastula	6
28	13.5	14.5-16	18-136	55	Xenopus laevis somatic cells (culture)	
23	15.5	10	10-144	66	"	
18	29.5	5-6	26-136	66	"	

1 - Yurov & Liapunova (1977)
2 - Huberman & Riggs (1968)
3 - McFarlane & Callan (1973)
4 - Callan (1972)
5 - Callan (1973)
6 - Callan (1976)

Taylor, 1968) and the initiation intervals, though not certainly known, are at least 1000 µm. Triturus neurula cells have initiation intervals in the order of 40 µm and an S-phase duration of 4-6 hrs. The cells of the Triturus blastula have initiation intervals less than 10 µm and an S-phase duration of about 1 hr. Therefore, reduction in the number of active initiation sites, as proposed by Callan, is likely to be primarily responsible for the lengthening of S-phase duration in the course of development.

Taylor (1974) has likewise suggested that the elevated number of initiation sites found in eukaryotic embryogenesis could wholly account for the rapid rate of DNA synthesis at this time. Grippo et al., (1976) have found that Xenopus laevis kidney cells and full grown oocytes both lack DNA polymerase I, an enzyme which is present in Xenopus embryos until gastrulation. It seems hardly likely that this enzyme is induced or reactivated in Xenopus tissue culture cells when these go through shortened S-phase at higher temperatures, for as I have shown the initiation intervals appear to be constant regardless of temperature.

Different tissues may well have different numbers of active sites in relationship to their differing S-phase durations (Callan, 1972; Hand & Tamm, 1974). Callan proposed that variation in DNA/histone packing within the chromosomes of different tissues might be responsible for the variation in the number of active origins. He pointed out, for example, that in Triturus spermatocyte nuclei where much of the chromatin is densely packed a smaller number of sites may be exposed to initiator enzyme(s) than in typical somatic nuclei where the DNA/ histone is more diffuse. If it be the case that DNA/histone packing does not change in Xenopus tissue culture cells over the range 18° to 28°C, then the number of exposed initiation sites should remain constant.

Variable staggering in the initiation times of active origins might be another factor responsible for variation in S-phase duration in the course of development or in different types of cells (Amaldi et al., 1973; Callan, 1973; McFarlane & Callan, 1973). Staggering of initiation times has been found for Xenopus cells at all three temperatures, 18°, 23° and 28°C and a particularly good example of this is shown in fig. 15.

If replication from an origin proceeded to a "terminus", the positions of such termini and their relationship to staggered initiations would be expected to affect S-phase duration. Termini were originally proposed by Huberman & Riggs (1968) but all later observers have concluded from the available evidence that termini do not exist (e.g. McFarlane & Callan, 1973; Hand, 1975). I have noticed only one fibre autoradiograph where a terminus might be construed to be present (fig. 8), but I should emphasize that this is a most exceptional case. By contrast, I have seen many examples of fibre autoradiographs which provide indisputable evidence against the existence of termini; this is particularly clear in the case of fig. 15.

The likeliest single factor responsible for decreasing S-phase duration with increasing temperature is the rate of progress of replication forks. Table 13 shows how S-phase durations and fork progression rates are related. If fork progression rate (FR) were alone responsible for different S-phase durations, then one would expect the product S x FR to be constant. For 18°C and 23°C the products are much about the same (162 and 155 respectively) but for 28°C the product is considerably larger, particularly so if the more reliable estimate of FR is taken (216). This suggests that temperature-dependent variable staggering in the activation of origins is another factor to be taken into account, though it be of less consequence than fork progression rate. Taking the evidence just as it stands, there would appear to be disproportionately more staggering of origins at higher than at lower

TABLE 13. The relationship between temperature, S-phase duration, and rate of progress of replication forks.

Temp. °C	S-phase duration hrs	Fork rate (FR) µm/hr	Product of S x FR
18	29.5	5.5	162
23	15.5	10	155
28	13.5	14.5 (less reliable)	196
		16 (more reliable)	216

culture temperature, i.e. the S-phase duration at 28°C is unexpectedly long drawn out. One needs to bear in mind that perhaps 28°C is somewhat above the well-tolerated temperature range of these cells.

Chibon's results (1973) show that in various tissues of Pleurodeles the lengths of the cell cycle phases are temperature-dependent. He proposed that the extension of S-phase at low temperature might be due to a decreased rate of production of enzymes and precursors necessary for DNA synthesis. Saladino & Johnson (1974) showed that in Chinese hamster cells S-phase is related to the rate of DNA synthesis and is a function of incubation temperature. The rate of DNA synthesis is 1.44×10^{-14} g/min/cell and the S-phase is 5 hrs at 39.5°C, whilst the DNA synthetic rate is 0.6×10^{-14} g/min/cell and the S-phase duration is 11.9 hrs at 31.5°C. It is to be noticed that here the two products 5 x 1.44 and 0.6 x 11.9 are much the same, 7.2 and 7.1 respectively. For these same cells we have the further information from Yurov & Liapunova (1977) that fork progression rates are 48 µm/hr at 37°C and 36 µm/hr at 31.5°C.

It needs to be stressed that my estimates of fork progression rates at the three temperatures are for the most part estimates of rates in operation early in S-phase, because I used culture conditions where partial synchrony had been produced by FUdR treatment prior to labelling. We do not know whether fork progression rates in later stages of the Xenopus S-phase are similar to those in early S or whether, as has been claimed by Housman & Huberman (1975) fork progression rate increases through the S-phase. These authors maintain that there is a 3-fold increase in fork progression rate in Chinese hamster cells from early to late S, though other interpretations of their data are possible.

Fork progression rate, the number of active sites, staggering in the sequence of initiation times, the rates of synthesis of proteins required as initiating factors and for chain elongation are all of consequence during the S-phase period. Also the concentrations of precursors, the relative diffusion rates of small and large molecules between the cell cytoplasm and the nucleus, and how these relate to pH and temperature, all govern the metabolism of the cell and therefore the duration of the S-phase. Studies carried out on mammalian cells have shown that the level of deoxyribonucleoside triphosphate pools are low during G_1 and increase steadily during S-phase (Skoog & Nordenskjold, 1971; Walter, et al., 1973). Inhibition of triphosphate uptake by treatment with hydroxyurea demonstrates the dependence of rate of DNA synthesis on triphosphate pool sizes (Skoog & Nordenskjold, 1971; Skoog & Bursell, 1974).

The synthesis of a specific acidic protein involved in the initiation of DNA synthesis has been shown in yeast cells, but that, once initiated, the S-phase proceeds without further protein synthesis being required (Hereford & Hartwell, 1973). In mammalian cells, it has been shown that protein synthesis in G_1 is a prerequisite for the onset of the S-phase period (Highfield & Dewey, 1972). Inhibition of protein synthesis in G_1 inhibits DNA replication (Weintraub & Holtzer, 1972; Hand & Tamm, 1973; Hori & Lark, 1973; Gautschi, 1974). At the present time it is not known whether inhibition of DNA synthesis results from the inhibition of polymerase synthesis or the synthesis of some other essential protein. Histones are unlikely to be involved directly in the initiation of DNA synthesis, for histones are themselves synthesised during DNA replication (Takai, et al., 1968); however the precise quantitative relationship between amount of histone and amount of DNA per nucleus indicate that the two syntheses are closely controlled and coupled. Swift (1962; 1964; 1965) has shown a constant

quantitative relationship between DNA and histone, not only throughout the cell cycle, but also between different states of chromatin packing. However the transcription of histone genes could conceivably be a key factor in the initiation of DNA synthesis, and the duration of synthesis, the rate of DNA synthesis being perhaps governed by the availability of histones, and therefore upon the rate of transcription of the histone genes, and on translation of histone messenger molecules. This does not take us closer to an understanding of the initiating mechanism; it merely displaces the question to what governs the transcription of the histones genes.

The enzymes which are specifically concerned in DNA synthesis appear to be synthesized with some periodicity, often during or just preceding the S phase (see, Mitchison, 1971). The slow rate of DNA replication and long S-phase duration consequent on culture at low temperature may be due not only to a reduction in the rate of protein synthesis but also because diffusion rates are lower at lower temperatures. Several studies have shown that proteins synthesized in the cytoplasm are subsequently accumulated by the nucleus (Bloch & Brack, 1964; Prescott & Goldstein, 1968; Speer & Zimmerman, 1968; Merriam, 1969). This is notably true of histones.

Prescott & Goldstein (1967) have shown that when a G_2 nucleus of an Amoeba is transferred into an S-period Amoeba, the transplanted G_2 nucleus reinitiates DNA synthesis. Conversely when an S-phase nucleus is transferred into a G_2 cell, DNA synthesis in the transferred nucleus is sharply reduced. This must mean that S-phase cytoplasm supplies initiator signals to the nucleus, and that G_2 cytoplasm either supplies inhibitors or alternatively, fails to supply the factors necessary for continuation of synthesis. De Terra (1967) has similarly shown in Stentor that a nucleus in G_2 initiates DNA replication when introduced into an S-phase cell, but if a nucleus

in S-phase is introduced into a G_1 cell, DNA synthesis ceases. This must again mean that the cytoplasm of an S-phase cell contains initiator signals, whereas the cytoplasm of G_1 cells lack initiators or contain inhibitors for DNA synthesis.

One might argue that the temperature-dependence of the durations of the cell cycle has a simple explanation, that the overriding factor is rate of diffusion. Diffusion rate is directly related to temperature, and it may be that at 18°C the diffusion rate of precursor molecules into the nucleus is substantially lower than at 23°C. However as the rate of fork progression at 18°C is roughly half that at 23°C, it would be difficult to argue this point convincingly. However, if lower temperatures significantly affect other basic metabolic processes in the cell, these may be manifested as osmolarity differences within the cell which may in turn substantially affect the diffusion constants.

But should one anticipate a prime role for any single factor with temperature change? The results presented here show that it is the rate of fork progression that is influenced by temperature. Whether or not this effect is a direct expression of the enzymes decreased mobility or whether it is because of one or several of the possible secondary effects, has yet to be determined. Certainly this is a key point in a comprehensive understanding of the cell cycle and its durations, and because of this it is a point central to the study of animal development.

SUMMARY

Pulse/chase labelling and DNA fibre autoradiography have been used to study the cell cycles of Xenopus cells in tissue culture (A-6 line) at 18°, 23° and 28°C.

1. At 18°C G_1 lasts for 31 hrs, S for 29.5 hrs, G_2 for 8.5 hrs M for 3 hrs, and the total generation time is 72 hrs.

2. At 23°C G_1 lasts for 14.3 hrs, S for 15.5 hrs, G_2 for 5.7 hrs, M for 0.5 hrs and the total generation time is 36 hrs.

3. At 28°C G_1 lasts for 11.3 hrs, S for 13.5 hrs, G_2 for 4.8 hrs, M for 0.4 hrs and the total generation time is 30 hrs.

4. Whole cell autoradiographs after brief ^{3}H-TdR labelling give, to a first approximation, a direct measure of the proportions of the cell cycle occupied by the various phases. The results are substantially in accord with the figures given in 1 to 3 above.

5. Chromosomal DNA has been shown to replicate bidirectionally from initiation sites or origins, and fork progression rates are 5.5 μm/hr at 18°C, 10 μm/hr at 23°C and 16 μm/hr at 28°C.

6. The ranges of and mean initiation intervals at all three temperatures are much the same, the means being of the order 60-66 μm.

7. Staggering of initiations is evident at all three temperatures, and may be disproportionately greater at 28°C than at the two lower temperatures.

8. Evidence is against the existence of replication termini.

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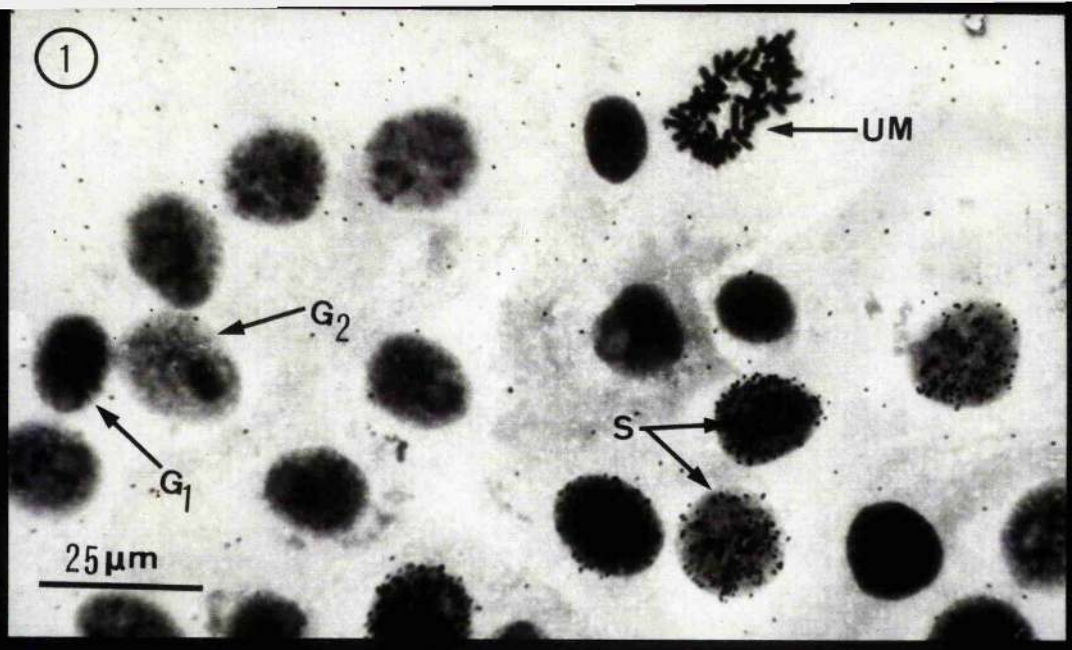
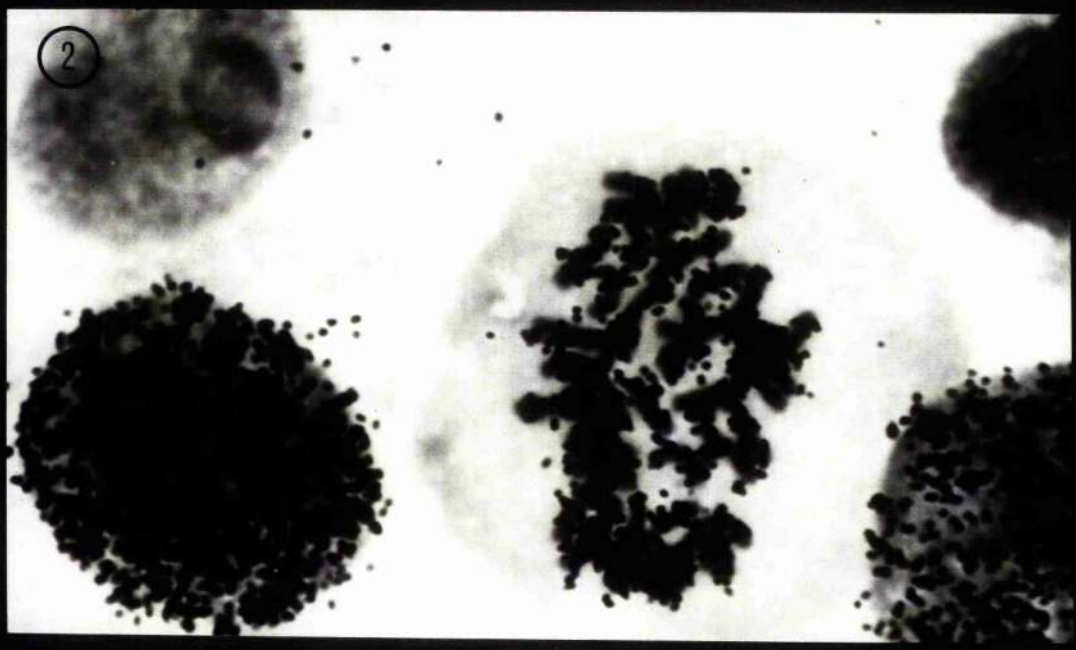
Plate 1.

Fig. 1 is a photograph of a whole-cell autoradiograph of Xenopus kidney cells in culture at 18°C, stained with Giemsa. The cells were labelled with ^{3}H-TdR for 1 hr and then fixed. This photograph shows that interphase cells fall into 3 groups: labelled cells are in S, small unlabelled cells in G_1 and large unlabelled cells in G_2. The unlabelled metaphase (UM) was in mitosis during the labelling period.

Fig. 2 is a photograph of a whole-cell autoradiograph from a culture which was labelled for 1 hr, then transferred to non-radioactive medium, and fixed 24 hrs later. It shows a labelled metaphase as well as 2 labelled and 2 unlabelled interphases.

Fig. 3 as Fig. 2 but the cells were fixed 90 hrs later. This is probably a cell which was in S during the presence of ^{3}H-TdR, passed through mitosis and another synthetic period, this time in absence of ^{3}H-TdR, and then fixed at the second mitosis.

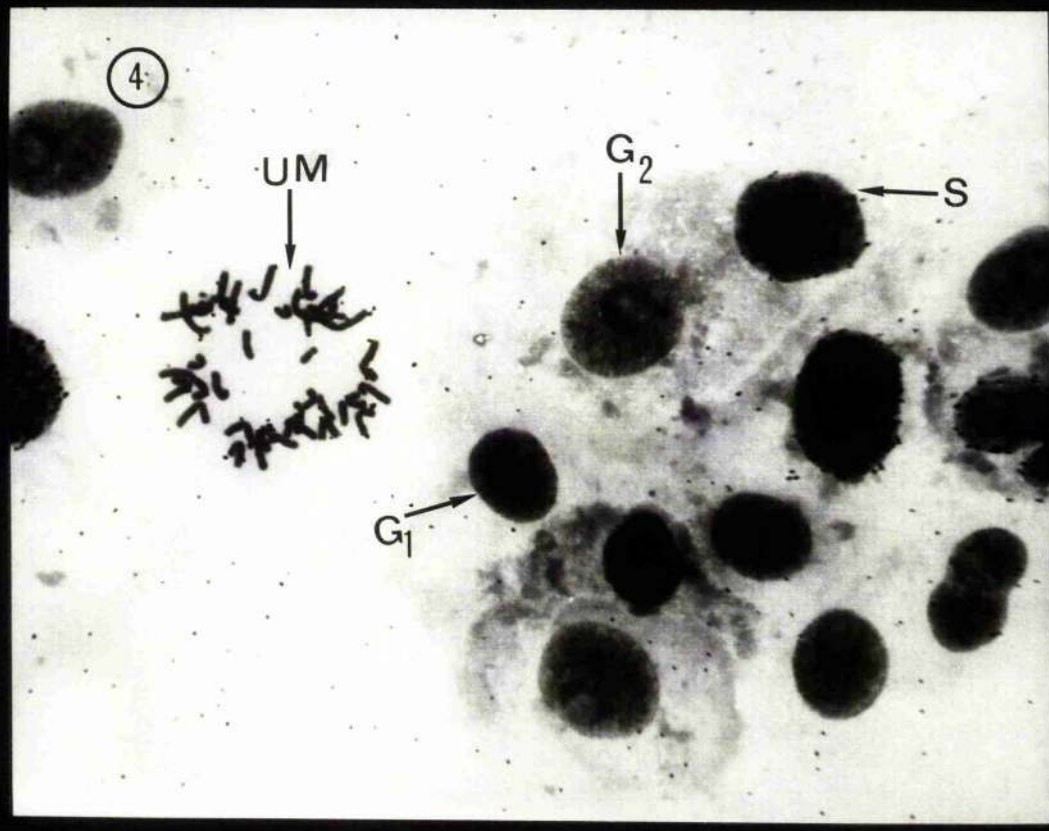

①
UM
G2
S
G1
25μm


②


③
125μm

Plate 2.

Figs 4 and 5 are photographs of whole-cell autoradiographs of Xenopus kidney cells in culture at 18°C, stained with Giemsa. The cells were labelled with ^{3}H-TdR for 1 hr, then transferred to non-radioactive medium, and fixed 24 hrs later. Both photographs show interphase cells falling into 3 groups: labelled cells (S) which were in S-phase at the time of labelling some of which may now be in G_2; small unlabelled cells in G_1, and large unlabelled cells in G_2. Fig. 4 includes an unlabelled metaphase (UM) which must have already reached G_2 when label was provided, while fig. 5 includes a labelled metaphase (LM) which must have been in S when label was provided.


4
UM
G2
S
G1

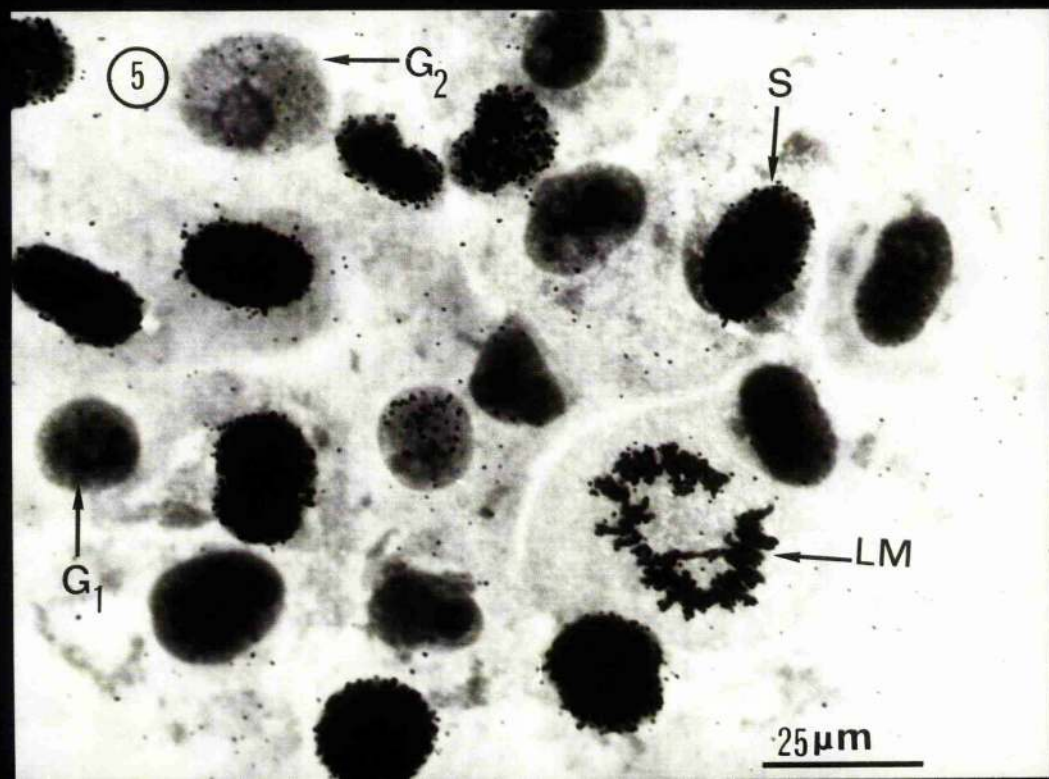

5
G2
S
G1
LM
25μm

Plate 3.

Figs 6 to 10 are photographs of DNA fibre autoradiographs prepared from Xenopus kidney cells (A-6) in culture at 18°C. The cultures were treated with FUdR and UR for 40 hrs, labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM) for 4 hrs, stepped down to ¼ specific activity and labelled for a further 4 hrs. The autoradiographs were exposed for 8 to 11 months.

Fig. 6 shows a track with 2 origins (1 & 2) which probably initiated replication at the beginning of labelling. Above and below there are "tails" which register replication occurring during the stepdown period, while in the middle 2 tails have converged, indicating fusion. There is an indication of sister strand separation at origin 2.

Fig. 7 shows a track with 2 nearly origins which initiated replication just prior to labelling. As in fig. 6, there are tails above and below, and fusion between adjacent tracks in the middle region which must have occurred before the stepdown.

Fig. 8 shows a track with 3 origins. Origin 3 had evidently initiated replication before labelling, origin 2 likewise though slightly later, while origin 1 had presumably initiated some time after the beginning of labelling. The short track above origin 2, which does not match the length of the divergent track below, is exceptional and unexplained.

Fig. 9 shows a track where 2 origins had already initiated replication, origin 2 somewhat earlier than origin 1. Above and below there are tails while fusion between adjacent tracks in the middle region must have occurred early in the stepdown period.

Fig. 10 shows a track where 2 origins had already initiated replication some 2 hrs before labelling. As in fig. 9 there are tails above and below, while in the middle 2 tails have converged, indicating fusion.

6
1
2
7
1
2
8
1
2
3
9
1
2
10
1
2
100μm

Plate 4.

Figs. 11 to 15 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in tissue culture at 23°C. The cultures were treated with FUdR and UR for 20 hrs, labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM) for 2 hrs, stepped down to ¼ specific activity and labelled for a further 2 hrs. The autoradiographs were exposed for 8 to 11 months.

Fig. 11 shows tandemly arranged tracks where 2 origins (1 & 2) had already initiated replication some time before labelling, and continued replication during the high specific activity and the stepdown periods. All 4 tracks show abrupt ends towards the inside, and well defined tails towards the outside, which register replication occurring during the stepdown period. The two tracks in the middle have reached, or nearly reached, fusion during the stepdown period.

Fig. 12 shows tandemly arranged tracks which had initiated replication during the period of labelling at high specific activity and continued replication during the stepdown period, as is shown by the tails of declining grain density at both ends of each track.

Fig 13 shows 4 tandemly arranged tracks which had initiated replication during the period of labelling at high specific activity and continued replication during the stepdown period. Except the rightmost track, about which there is some uncertainty, these tracks all show tails at both ends, and fusion has evidently occurred between the two tracks in the middle during the stepdown.

Fig. 14 shows 4 tandemly arranged labelled tracks which had initiated replication during the period of labelling at high specific activity, and continued replication during the stepdown period. Each track has tails at both ends.

Fig. 15 shows a tandem series of tracks where 3 origins (1, 2 & 3) had already initiated replication before label was provided. Origin 1 had initiated replication before 2 and 3, while origin 3 had initiated before 2. The two tracks above and below at the outside show abrupt inside ends, and well defined tails towards the outside. The two tracks in the middle, which show sister strand separation, terminate abruptly at both ends, and represent the outcome of fusions between the replication units 1 and 2 and between 2 and 3, both of which must have occurred before the stepdown period.


11
1
2
12
13
14
15
1
2
3
100 µm

Plate 5.

Figs. 16 to 23 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in tissue culture at 18°C. The cultures were treated with FUdR and UR for 40 hrs and then labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM). The autoradiographs were exposed for 8 to 11 months.

Figs 16 to 18 show tandemly arranged tracks from cultures labelled for 2 hrs.

Figs. 19 to 21 show tandemly arranged tracks from cultures labelled for 4 hrs.

Figs. 22 to 23 show tandemly arranged tracks from cultures labelled for 8 hrs.

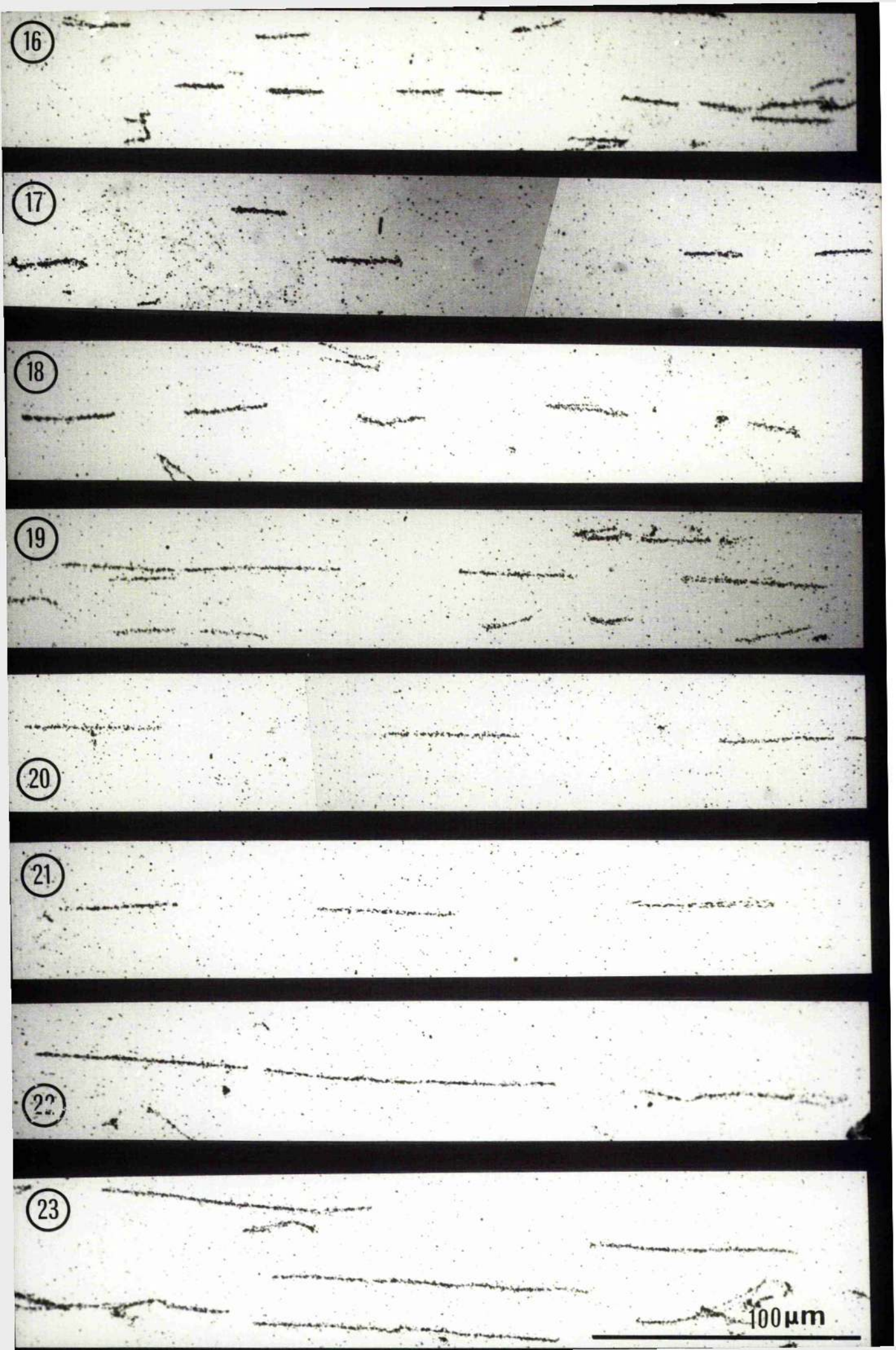

16
17
18
19
20
21
22
23
100μm

Plate 6.

Figs. 24 to 27 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in tissue culture at 18°C. The cultures were treated with FUdR and UR for 40 hrs and then labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM) for 8 hrs. The autoradiographs were exposed for 8 to 11 months.

Fig. 24 shows 2 tracks which because of their lengths are probably the result of fusions between neighbours.

Fig. 25 shows 3 tracks all of whose lengths suggest that fusion between neighbouring units has occurred, and all 3 are evidently close to fusion with one another. Arrows indicate where sister strand separation has occurred.

Figs. 26 and 27 show tracks which form parts of a continuous tandem series, and are remarkable for the exceptional distance between presumed neighbouring origins.


24
25
26
27
100µm

Plate 7.

Figs. 28 to 35 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in culture at 23°C. The cells were treated with FUdR and UR for 20 hrs and then labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM). The autoradiographs were exposed for 8 to 11 months.

Figs. 28 to 32 show tandem series of tracks from cells labelled for 2 hrs.

Figs. 33 to 35 show tandem series of tracks from cells labelled for 4 hrs.

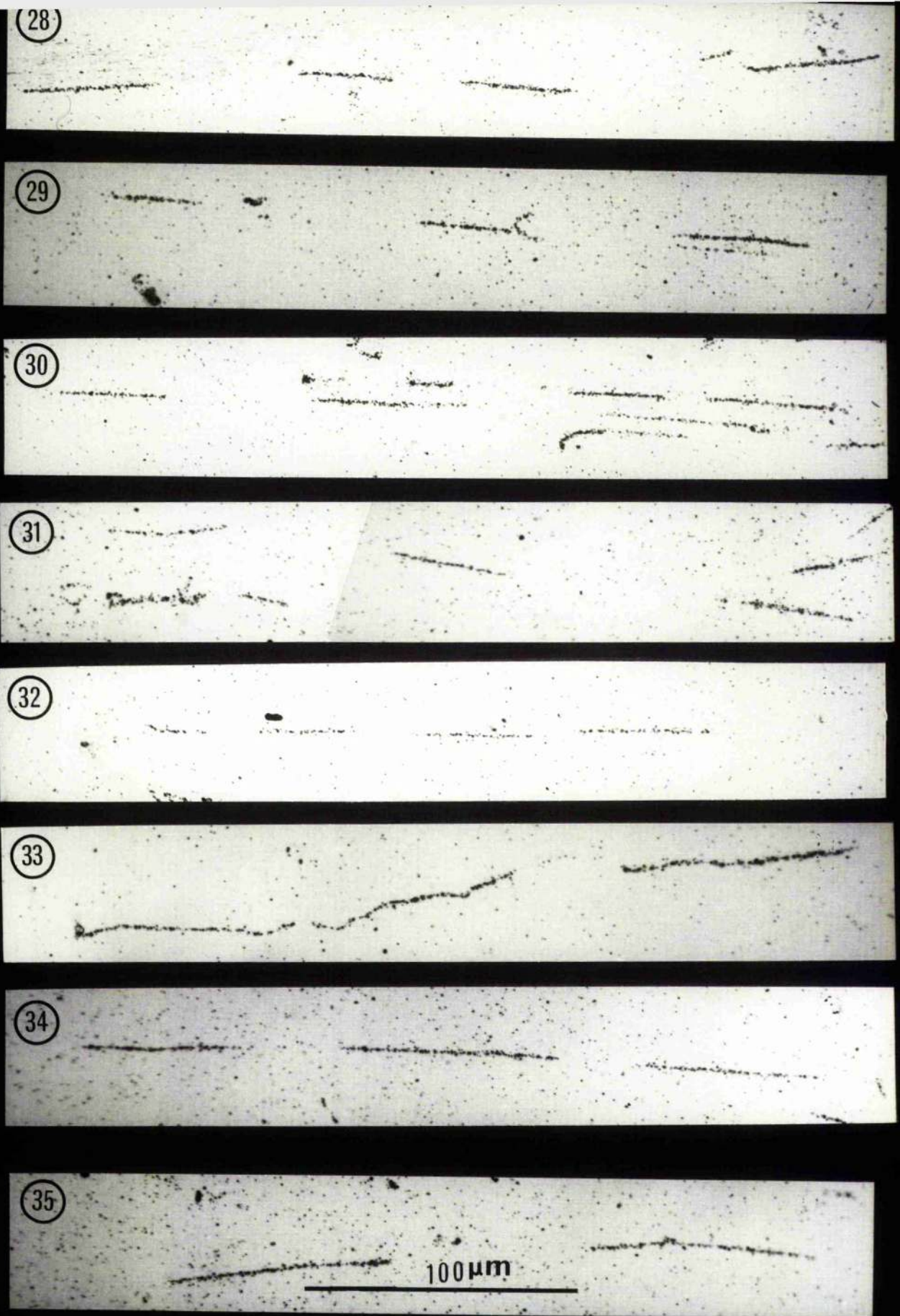

28
29
30
31
32
33
34
35
100μm

Plate 8.

Figs. 36 to 40 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in culture at 23°C. The cultures were treated with FUdR and UR for 20 hrs and then labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM) for 4 hrs. The autoradiographs were exposed for 8 to 11 months.

The lengths of these tracks are roughly twice as long as those present in autoradiographs from cells which were labelled for only 2 hrs.

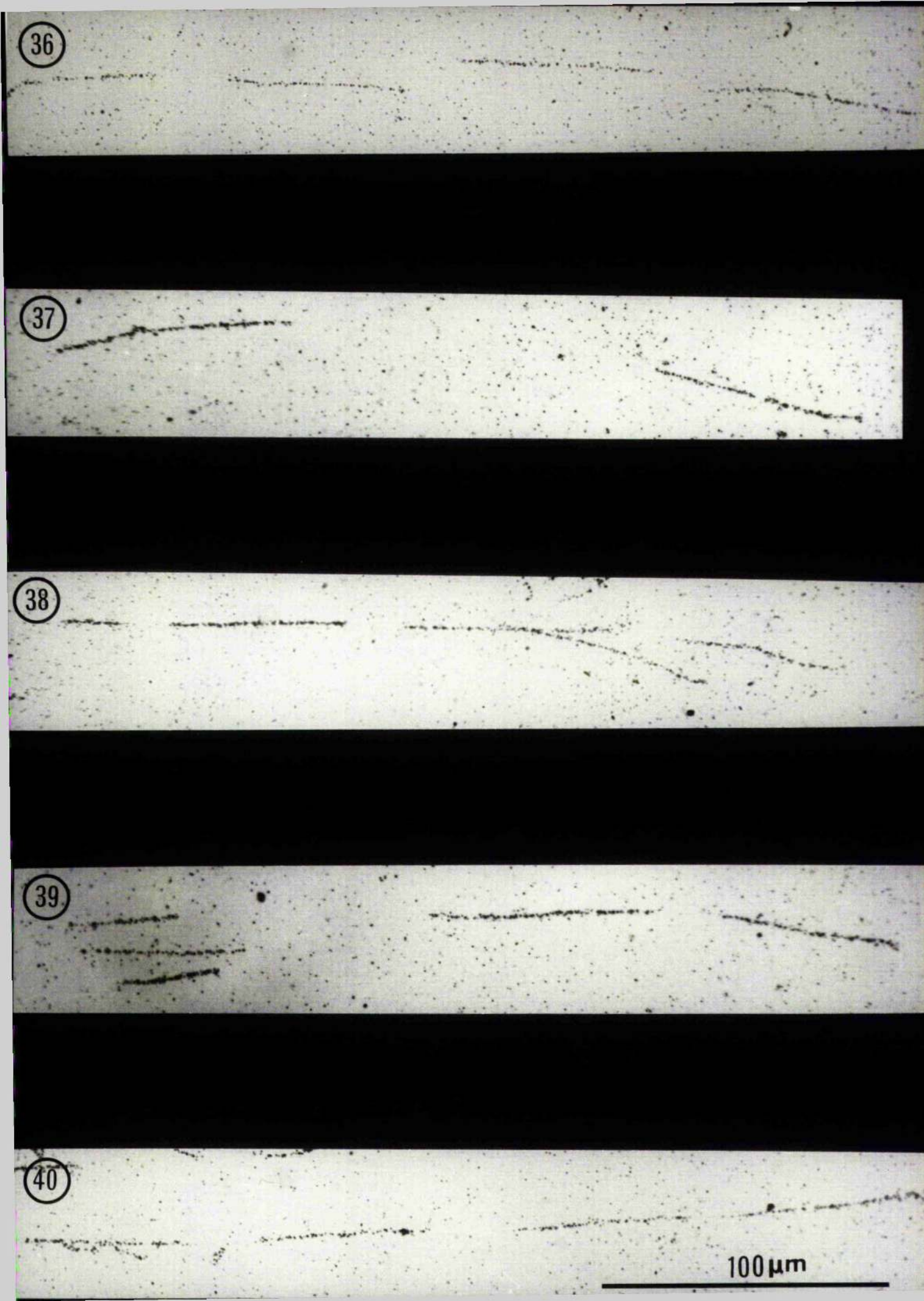

36
37
38
39
40
100 µm

Plate 9.

Figs. 41 to 48 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in culture at 28°C. The cultures were treated with FUdR and UR for 20 hrs and then labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM). The autoradiographs were exposed for 8 to 11 months.

Figs. 41 to 43 show tandem series of tracks from cultures labelled for 1 hr.

Figs. 44 to 46 show tandem series of tracks from cultures labelled for 2 hrs.

Figs. 47 to 48 show long uninterrupted tracks, resulting from fusion, coming from cultures labelled for 4 hrs. Arrows in fig. 48 indicate where sister strand separation is apparent.

Plate 10.

Figs. 49 to 52 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in culture at 28°C. The cells were treated with FUdR and UR for 20 hrs and then labelled with ^{3}H-TdR (50 µC/ml, 26 Ci/mM) for 4 hrs. The autoradiographs were exposed for 8 to 22 months.

All 4 photographs show some tracks which, because of their lengths, must have resulted from fusions during the labelling period. The arrow in fig. 50 marks where sister strand separation is apparent.

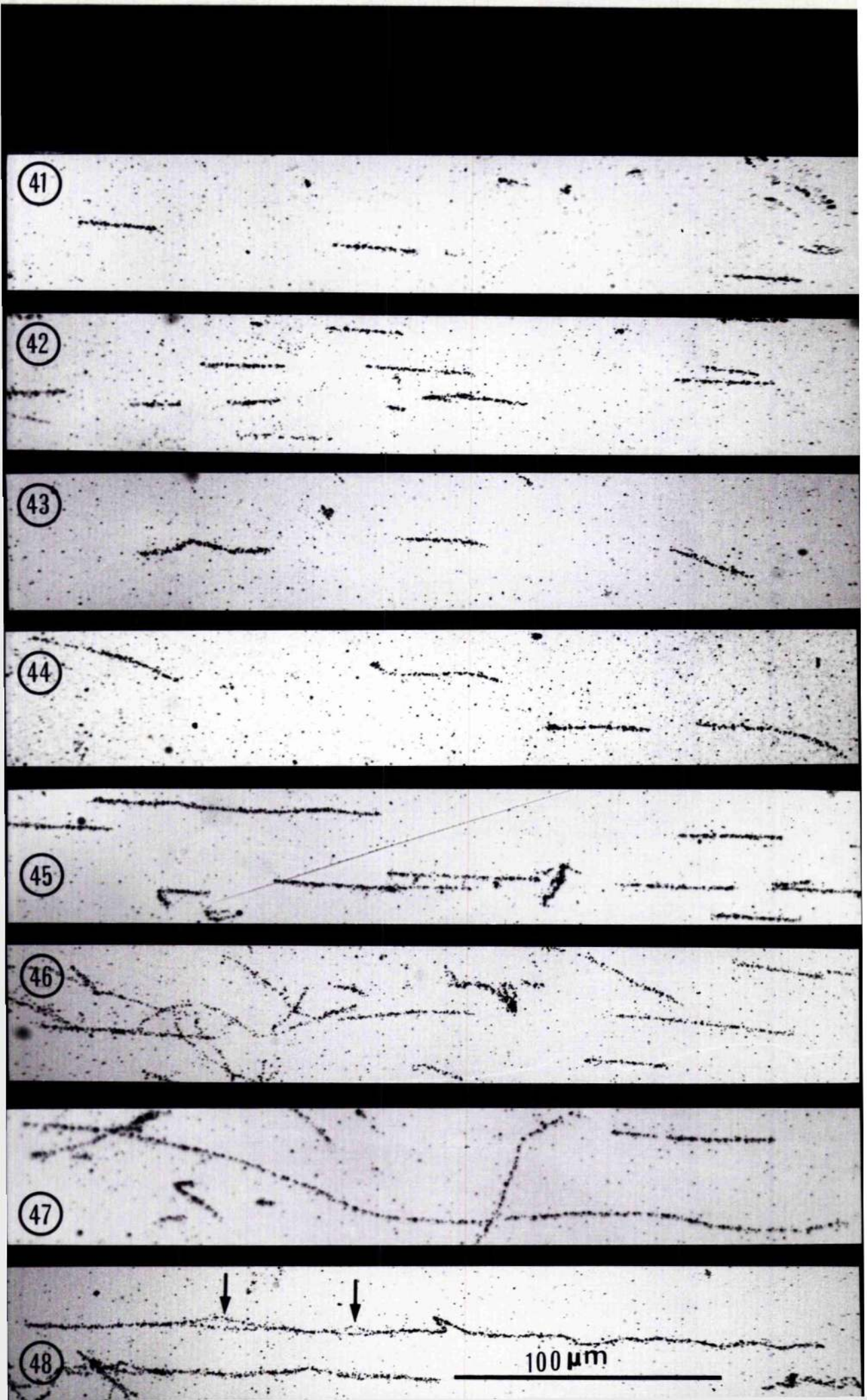
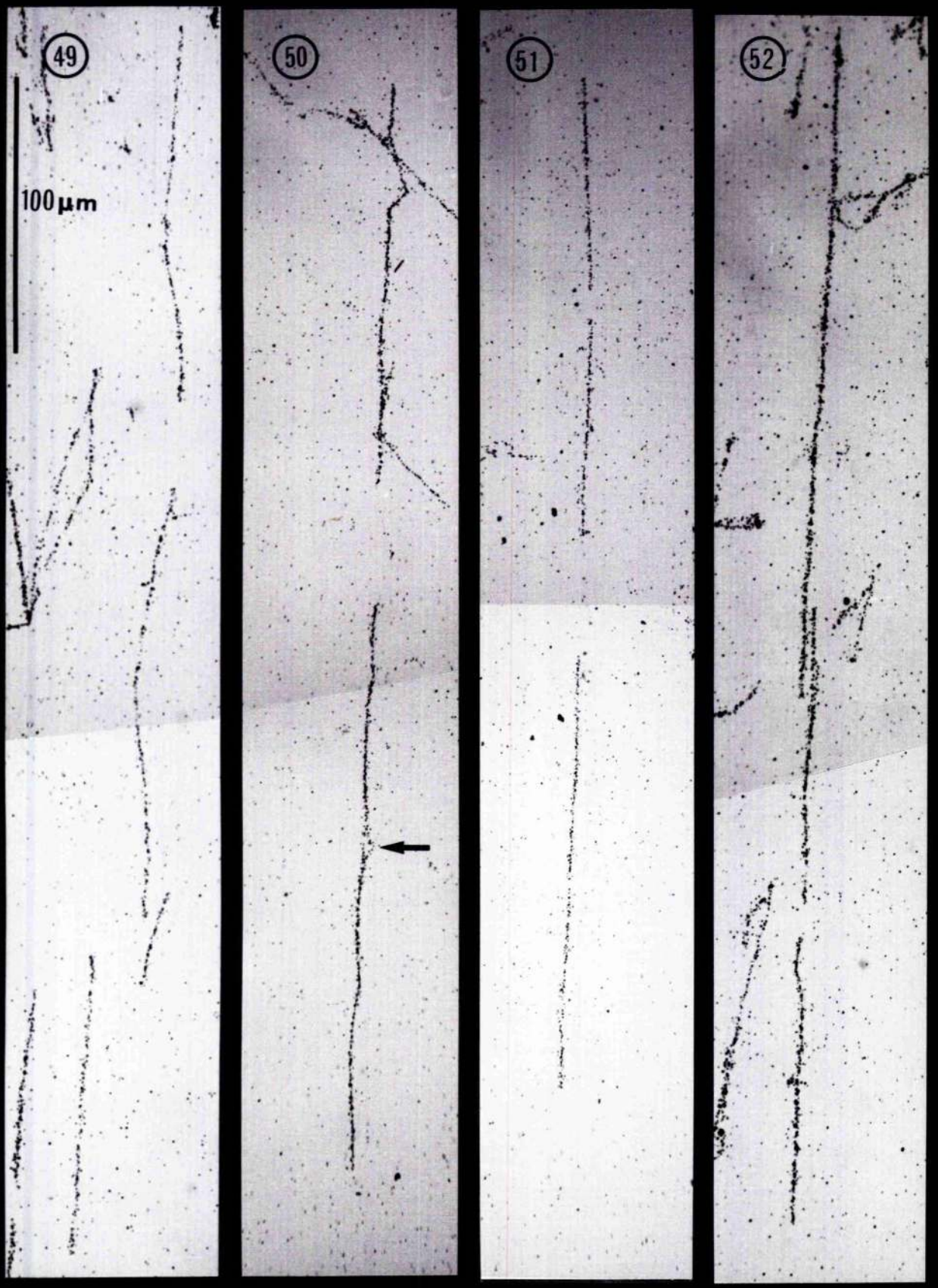

41
42
43
44
45
46
47
48
100 μm

Plate 11.

Figs. 53 to 55 are photographs of DNA fibre autoradiographs prepared from Xenopus cells in tissue culture, and chosen as being typical of tracks resulting from cultures labelled for 2 hrs at 18°C (53), at 23°C (54) and at 28°C (55). Labelling was in all cases preceded by FUdR treatment, and the exposure times were 8 to 11 months. The three photographs show clearly how track lengths are dependent on culture temperature, provided the time of labelling is chosen sufficiently short as not to permit fusions to occur between replicating segments which initiated during the labelling period.


49
100 µm
50
51
52


53


54

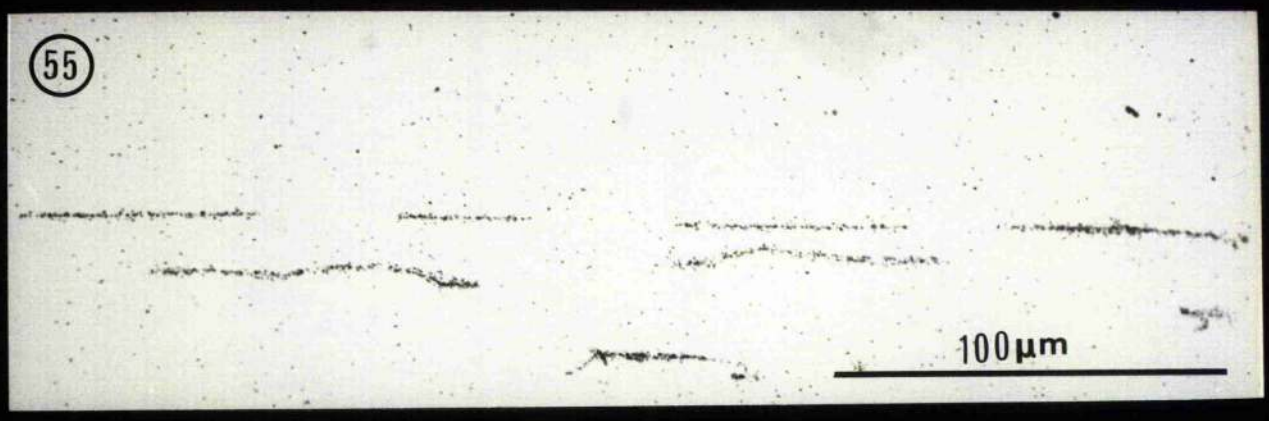
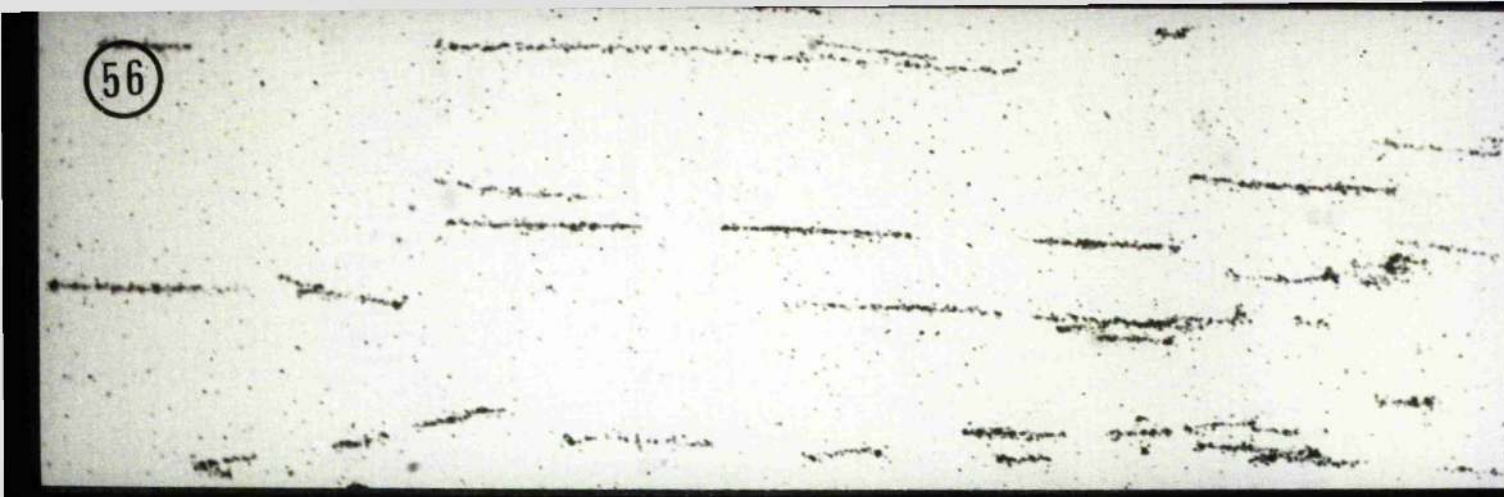

55
100µm

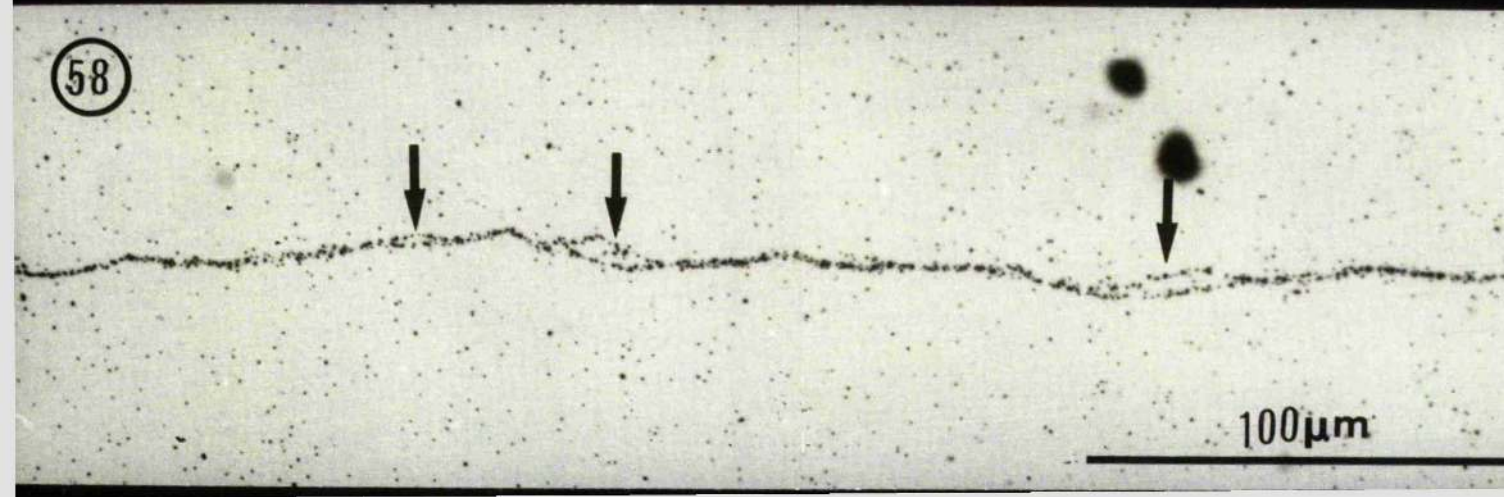
Plate 12.

Figs. 56 to 58 are photographs of DNA fibre autoradiographs prepared from *Xenopus* cells in tissue culture, and chosen as being typical of tracks resulting from cultures labelled for 4 hrs at 18°C (56), at 23°C (57) and at 28°C (58). Labelling was in all cases preceded by FUdR treatment, and exposure times were 8 to 11 months.

The long track at the top of fig. 56 is somewhat unusual: it certainly represents fusion. The very long track in fig. 58, where arrows indicate sister strand separation, is entirely typical of fusions obtaining after 4 hrs labelling at 28°C.


56


57


58
100µm